THE AUTONOMIC NERVOUS SYSTEM: ITS INFLUENCE ON THE IMMUNE SYSTEM AND DISEASE

By

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

THE AUTONOMIC NERVOUS SYSTEM: ITS INFLUENCE ON THE IMMUNE SYSTEM AND DISEASE

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The premise of this project is that the sympathetic nervous system (SNS) can act on the immune system to increase susceptibility to disease states. We were able to quantify different lymphocyte subpopulations in different experimental and genetic rat models of increased SNS activity using fluorescence activated cell sorting (FACS) analysis. The lymphocyte subpopulations examined were the T-cells, B-cells and two T-cell subgroups, the T-helper cells and the T-nonhelper cells. The initial experiment involved injecting two peptides, angiotensin II and substance

P, that increase SNS activity, into the brains of intact rodents. The results showed that the animals receiving the peptide infusions had an increased percentage of T-cells in their peripheral blood. Another series of experiments involved two genetic models of increased SNS activity, the spontaneously hypertensive rat (SHR) and the Dahl S/JR rat. Both of these strains showed decreases in the percentage of the T-nonhelper population in the prehypertensive as well as hypertensive phases of development. Guanethidine treatment of neonatal SHR was undertaken to destroy the peripheral sympathetic nervous system. These guanethidine treated SHR had normalized blood pressure and a T-nonhelper cell percentage in line with the WKY control animals. Interleukin-2, a powerful T-cell proliferation factor, had no effect on the blood pressure of the SHR but did increase the T-nonhelper cell percentage at one time point. Another important finding of this project was the high levels of angiotensin II, an important neuropeptide and peripheral vasoconstrictor, found in the spleens of three rat strains. The immune system has been implicated in the pathogenesis of hypertension in both man and experimental animals, such as the SHR. In man, another pathological process, periodontal disease, also is believed to have an immune component. The mandibles of SHR were found to have increased bone and tooth loss. The studies indicate that the brain influences the immune system through the sympathetic nervous system which can be activated by a brain peptide such as angiotensin II. These results are relevant to our understanding of the pathogenesis of disease.

CHAPTER 1
INTRODUCTION

Hypothesis

The autonomic nervous system (ANS) regulates and modifies the immune response, thus altering the susceptibility to certain disease states. Increased sympathetic nervous system (SNS) activity, such as occurs in stress situations, leads to alterations in the immune system. These changes can then result in the development of disease states such as hypertension or periodontal disease.

The idea that stress affects our health is an old one. It is only relatively recently that we have been able to obtain experimental evidence concerning the mechanism behind this observation. Selye (1970) was among the first to note alterations in the immune system in response to stress. This thymicolymphatic atrophy was just one of the responses he observed during the development of his concept of a stereotyped response to stress. More recently there has been an enormous amount of data generated linking the central nervous system and the immune system.

The overall aim of our project is to more closely examine the role of the ANS in regulating and modifying the immune system and then study how this interaction may be involved in the development of certain disease states such as hypertension and oral disease. The focus will be on the actions of the sympathetic portion of the ANS.

The following four specific aims provide a structure from which to examine certain aspects of this question.

Aim One: To Determine the Effect of Peptides that Increase Sympathetic Output on Specific Peripheral Immune System Components

Angiotensin II (Ang II) has been shown to increase sympathetic output (Printz and Lewicki, 1977; Aars, 1977; Boadle-Biber and Roth, 1977; Samuels et al., 1977; Severs et al., 1971; Severs and Daniels-Severs, 1973; Unger et al., 1981). These studies have involved Ang II in the periphery and centrally. The peripheral Ang II stimulation of the sympathetic nervous system (SNS) is brought about mainly by actions on sympathetic ganglia (Feldberg and Lewis, 1964), adrenal medulla (Feldberg and Lewis, 1964; Reit, 1972) and post-ganglionic enhancement of catecholamine (CA) release (Zimmerman et al., 1972). Intravenous injection of Ang II has also been shown to produce an increase in renal nerve activity (Aars, 1977). Several investigators report an increase in SNS activity to centrally administered Ang II (Printz and Lewicki, 1977; Aars, 1977; Severs et al., 1971; Severs and Daniels-Severs, 1973; Unger et al., 1981). The exact involvement of the various peripheral and central components of the SNS are not well defined (Unger et al., 1981). Generally, plasma CA levels are considered a reliable measure of SNS activity (Unger et al., 1981). When Ang II is given directly into the brain, plasma CA levels increase (Unger et al., 1981). In addition to this CA effect, it is known that following intracerebroventricular (ICV) injection of Ang II, arginine vasopressin (AVP) and adrenocorticotropic hormone (ACTH) levels rise (Severs and Daniels-Severs, 1973; Reid and Day, 1977). In the brain itself, centrally injected Ang II appears to stimulate norepinephrine (NE)-rich

nuclei in the brain stem and hypothalamus (Sumners and Phillips, 1983; Ganten et al., 1980) but does not alter dopamine. Also, Ang II injected ICV appears to cause the release of NE into the cerebrospinal fluid (CSF) (Chevillard et al., 1979) and increase CA turnover in the brain stem (Garcia-Sevilla et al., 1979). Ang II stimulates the release of [3H]-NE (Schactt, 1984). The peripheral portion of the SNS that mediates the cardiovascular effects of centrally injected Ang II is also activated but the relative contribution of this component to the pressor action has not yet been fully clarified (Unger et al.,1981). There is evidence that AVP release into the circulation acts along with this peripheral stimulation of the SNS to participate in the cardiovascular increase to central Ang II (Severs and Daniels-Severs, 1973; Unger et al., 1981; Hoffman and Phillips, 1977) and that these two factors act in parallel. The exact beds which are constricted by Ang II sympathetic activation are not yet clear although total peripheral resistance is increased.

Substance P (Sub P) is another peptide which increases sympathetic outflow when injected centrally. Substance P is a blood pressure regulating neuropeptide that is localized in brain areas involved in cardiovascular control such as the medulla oblongata and hypothalamus (Hokfelt et al., 1978; Gillis et al., 1980). Evidence indicates that there is an increase in blood pressure following injection into the ventricular system of the brain (Traczyk and Kubicki, 1980). These Sub P pressor effects are less potent than those of Ang II, but have a longer duration (Unger et al., 1981). Following ICV injection of Sub P there appears to be a rise in plasma NE levels (Unger et al., 1981). This can be considered as an index of increased sympathetic activity at nerve endings (Yamaguchi and Kopin,

1979) and thus, an indication of an increase in peripheral SNS outflow. There appears to be no simultaneous increase in AVP as occurs with Ang II (Unger et al., 1981). There also appears to be some evidence to suggest that central injection of Sub P causes more sympathetic activation than seen after central injection of Ang II (Unger et al., 1981). In summation then, ICV Sub P appears to activate the peripheral portion of the SNS more intensely than Ang II.

The purpose behind injecting peptides into the brains of intact rodents is to produce an increase in sympathetic outflow and then measure the resultant changes in various immune parameters. There is already quite a large body of evidence suggesting that stress and/or altered ANS activity can change the immune response and contribute to disease states (Davis and Jenkins, 1962; DeMarco, 1976; Manhold and Weisinger, 1971; Shields, 1977; Davis, 1984; Workman and La Via, 1987; Marx, 1985). By experimentally producing an increase in sympathetic activity, it is hoped that an adequate model will be developed to better study the relationship between the ANS, the immune response and their interaction in mediating various disease states not only in animals but in man as well. This, in turn, will provide more clues to how environment and behavior influence health. There is already much evidence of this link in the body as a whole as well as specifically in the oral cavity (Davis and Jenkins, 1962; DeMarco, 1976; Shields, 1977; Davis, 1984; Workman and La Via, 1987; Marx, 1985; Haskell, 1975) which will be discussed later. Thus, the autonomic nervous system may be a mirror of emotional health. It has been demonstrated that the autonomic nervous system sends fibers to the thymus gland as well as other immune organs. These nerves tend to aggregate in areas rich in T-cells and avoid areas where developing B-cells

are present (Bulloch, 1985). It has already been shown that an increase in sympathetic activity is associated with a decrease in immune response (Cross et al., 1985; Braun et al., 1985; Ader, 1981). It is interesting to note that a genetically pure animal model exists that demonstrates many of the previously mentioned connections between altered autonomic activity and the immune response. The spontaneously hypertensive rat (SHR) has been shown to have an overactive sympathetic nervous system (Norman and Dzielak, 1986; Guyton et al., 1974; Iriuchijima, 1973; Judy et al., 1976; Baird, 1977; Norman and Dzielak, 1982). There are some provocative data showing that these rats exhibit altered T-cell and B-cell ratios as well as other, more subtle alterations in their immune systems (Norman and Dzielak, 1986; Takeichi et al., 1986). They also have many indicators of increased brain angiotensin activity (Phillips and Kimura, 1986). This T-cell/B-cell ratio is one of the factors that is important in maintaining the integrity and proper function of the immune system. It is our wish to replicate this model experimentally to more closely examine the mechanism behind and action of this altered autonomic response. By changing the B-cell and T-cell ratios, many aspects of the immune response are altered affecting a variety of body functions. An example of one piece of this puzzle is the action of lymphokines. Lymphokines not only act locally (Grant et al., 1979a) in the immune response but are capable of acting centrally as well (Marx, 1985; Blalock, 1984; Blalock et al., 1982; Smith and Blalock, 1985). These lymphokines, such as ACTH- like substances (Blalock et al., 1982; Smith and Blalock, 1985) and interleukin, interferon-like substances (Marx, 1985; Blalock, 1984; Blalock et al., 1979), are capable of feeding back and acting on various brain areas to further

modulate the autonomic and immune responses. It is interesting to note that macrophages, which aid in T-cell activation, have been shown to contain angiotensin converting enzyme (Simon et al., 1986) although it is unclear exactly how this relates to the renin-angiotensin system's role in modulating the immune response. These interactions also strengthen the case for a connection between immune function and CNS function. By the use of these peptides administered into rat brains, we hope to be able to better quantify the effect of the ANS on immune components (T-cells, B-cells, T-cell subsets).

Aim Two: To Examine the Role of the Immune System, the Renin-Angiotensin System (RAS) and the Autonomic Nervous System (ANS) in the Development of Hypertension in the Spontaneously Hypertensive Rat (SHR)

Angiotensin converting enzyme (ACE) inhibitors are used clinically for the reduction of blood pressure, but their exact mechanism of action is not yet fully understood (Unger et al., 1983). It has been shown that chronic administration of ACE inhibitors lowers ACE activity levels in brain, aorta and kidney. The action of ACE inhibitors on lung and plasma ACE activity has not been as clear (Ikemoto et al., 1986; Moursi et al., 1986). Plasma renin activity increases with age in stroke prone spontaneously hypertensive rats (SHR-SP) (Matsunaga et al., 1975) and angiotensin II levels in the brain of the SHR are higher than control values (Phillips and Kimura, 1986). Along these same lines, it is interesting to note that Wilson et al. (1988) have shown that chronic treatment with the ACE inhibitor Captopril will reduce Ang II receptor binding in the brain of the SHR. Norman and Dzielak (1986), as well as others (Judy et al., 1976), have demonstrated that the SHR's sympathetic nervous system activity is

increased relative to controls. Several experiments have shown that ACE inhibitors will reduce hypertension in the SHR (Ikemoto et al., 1986; Moursi et al., 1986; Cohen and Kurz, 1982; Unger et al., 1985), but the exact mechanism of this action is still the topic of much debate.

The spleen is an important organ in the immune system. It filters blood in much the same way as lymph nodes filter lymph. During an episode of infection the spleen is one of the sites of lymphocyte proliferation and differentiation. Distinct populations of macrophages and other immunocompetent cells are also found in the spleen. Castren et al. (1987) have shown that there is considerable binding of Ang II in the spleen. This binding tends to be localized in the red pulp of this organ. Ang II binding has been found on lymphocytes (Shimada and Yazaki, 1978) and macrophages (Thomas and Hoffman, 1984; Weinstock and Kassab, 1984). Ang II has an inhibitory action on leukocytes probably caused by a stimulation of T-suppressor cells (Simon et al., 1986). Macrophages also contain angiotensin converting enzyme, indicating that Ang II may have significance in immune function (Hinman et al., 1979). Given this information, it would be useful to determine what levels of Ang II exist in the spleen.

Immune abnormalities have been suggested as a causative factor in both oral disease and hypertension. The connection between hypertension and immune abnormalities in both humans and spontaneously hypertensive rodents has been repeatedly documented (Norman and Dzielak, 1986; Khraibe et al., 1984; Bendich et al., 1981; Svendsen, 1979; Munro, 1978; Kirstensen, 1979, Olsen et al., 1973). The SHR exhibits an increased sympathetic nerve activity (Norman and Dzielak, 1986; Judy et al., 1976) and several investigators have linked this

to changes in the immune system (Norman and Dzielak, 1986; Judy et al., 1976; Takeichi et al., 1986; Khraibe et al., 1984; Takeichi et al., 1981; Fernandes et al., 1986). These changes involve alterations in the B-cell, T-cell ratio which appears to be significant in the pathogenesis of hypertension (Bendich et al., 1981). The T-suppressor cells in the SHR are especially depressed (Norman and Dzielak, 1986). However, no data are available as to when these changes occur and how they relate to the development of hypertension. We intend to look at these lymphocyte populations in both the prehypertensive and hypertensive phases of development. The T-suppressor cells are an important regulator of lymphokine secretion. B-lymphocytes, like T-lymphocytes, are capable of lymphokine secretion (Grant et al., 1979a). Lymphocytes can also secrete as a lymphokine an interferon-like substance as well as an ACTH-like substance that will cause adrenal cortisol secretion (Blalock, 1984). The role of the altered B-cell, T-cell ratio and resultant changes in lymphokine secretion in the SHR is unclear. The immunologic defects in the SHR in many ways parallel those found in human hypertensive conditions (Norman and Dzielak, 1986; Svendsen, 1979; Munro, 1978; Kirstensen, 1979; Olsen et al., 1973). These defects may trigger hypertension by causing vascular inflammation (Suzuki et al., 1978) or renal glomerular damage (Evan et al., 1981). The increased sympathetic activity may also play a role by increasing circulating catecholamines (Borkowski and Quinn, 1984). Another possible mechanism may involve direct action of lymphokines on the CNS and periphery.

A recent study by Tuttle and Boppana (1990) showed that when interleukin-2 (IL-2) is given to prehypertensive SHR the development of

hypertension is abolished. A decrease in blood pressure was also seen in adult SHR given IL-2. IL-2 is released by stimulated T-lymphocytes and causes the proliferation of activated T-cells. Both T-helper cells and cytotoxic T-cells are affected. The results of their expeiment are strengthened by data from others showing that there is an impairment and depression of T-lymphocyte function in the SHR (Takeichi et al., 1986; Takeichi et al., 1981). Also transplants of thymic tissue from Wistar Kyoto (WKY) rats into neonatal SHR can attenuate the development of hypertension (Norman and Dzielak, 1986). The thymus gland is involved in the development of T-cells so there is a precedent for this finding that IL-2, a strong T-cell activating factor, could affect the development of hypertension in this model. A goal of this project is to repeat this study and see if it affects the proportions of the different lymphocyte populations.

In order to more fully explore the influence of the ANS, specifically the SNS, on immune activity it would be beneficial to block the SNS and examine any resultant changes in the immune system. Guanethidine, when given from birth , produces a peripheral sympathectomy (Johnson et al., 1975). We intend to use this as an experimental model of reduced SNS activity and then measure any resultant changes in immune parameters. As has been mentioned previously, there is much evidence to suggest that SNS innervation to immune organs can alter immune cell ratios as well as immune function. SHR exhibit increased sympathetic activity as well as altered immune characteristics. By blocking this activity with guanethidine a better understanding of the ANS and its regulation of immune function can be obtained.

Aim Three: To Look at the Relationship Between the Occurance of Alterations in the Immune System and the Development of Hypertension in Different Rodent Hypertensive Models

It has already been shown that there is evidence of a link between the immune system and the development of hypertension in humans (Svendsen, 1979; Munro, 1978; Kirstensen, 1979; Olsen et al., 1973) as well as animals such the SHR (Norman and Dzielak, 1986; Unger et al., 1985; Khraibe et al., 1984; Bendich et al., 1981). In order to more carefully look at the immune system as one of the causative factors in hypertension, different hypertensive models should be examined. Other rodent models of hypertension have been left largely unexplored. The inbred Dahl rat strains are another genetic model of hypertension (Rapp and Dene, 1985). The original strain produced by Dahl was found not to be genetically pure. From the original Dahl strain, however, a genetically pure form has been developed (Rapp and Dene, 1985). The two strains, the Dahl salt sensitive (S/JR) and the Dahl salt resistant (R/JR), show different susceptiblities to sodium induced hypertension. When fed a high sodium diet, the S/JR rats will develop a severe hypertension that does not develop in the R/JR animals fed the same diet. Importantly, the S/JR will develop hypertension when on a normal sodium diet although not as rapidly (Rapp and Dene, 1985). These animals exhibit alterations in their CA usage (Kuchel et al., 1987; Racz et al., 1987) and also show the same type of vascular inflammation as that seen in the SHR (Rapp and Dene, 1985; Suzuki et al., 1978). By examining any immune system alterations that may occur in these animals, it is possible that a better understanding of their hypertension as well as of hypertension in general will develop.

Aim Four: The Relationship Between the ANS and the Development of Hypertension on Changes Occuring in the Oral Cavity

A further aim of our study is to look at the direct effects of the autonomic nervous system and the development of hypertension on the oral cavity in order to better understand the relationship and characteristics of the involved mechanisms. Alveolar bone and tooth loss are the parameters that will be examined. Although many studies have looked at the hypertensive characteristics of the SHR, we have not been able to find any work which has been done on the oral cavities of the SHR. As has been stated previously, the SHR has an overactive sympathetic nervous system. Normally, most blood vessels, including those of the periodontium, or supporting structures of the tooth, are controlled by sympathetic vascular tone with the tissue cells themselves playing a role via local humoral factors (Grant et al., 1979a). In the case of the chronic inflammatory process associated with the majority of periodontal diseases, these normal control mechanisms are again in operation, but to differing extents with their exact role not as clearly understood. The typical inflammatory response, including that associated with periodontal disease, can be initiated by both cellular and humoral immunologic reactions (Hyman and Zeldow, 1963; Rizzo and Mitchell, 1966; Ranney and Zander, 1970) as well as directly by bacterial endotoxins and enzymes (Grant et al., 1979a). Both of these processes can also activate the complement system which is capable of inducing inflammatory effects on its own (Grant et al., 1979a; Mergenhagen, 1970). Inflammation begins with a transient vasoconstriction followed by a prolonged vasodilation (Bhaskar, 1977; Rocha de silva and Leme, 1972; Zweifach, 1973). Following vasodilation, blood flow at first quickens, then slows to a more static pace

because of 1) increased viscosity of blood due to a loss of fluid from the vascular compartment (brought about by an increase in hydrostatic pressure produced by a lag in venular dilation) and 2) a vascular permeability increase due to the presence of protease systems (Spragg, 1974; Erdos, 1968) and lymphokines (Grant et al., 1979a). During the late phase of the inflammatory process, sludging and thrombus formation may occur from fibrin formation brought about by platelet aggregation. If severe enough, these vascular changes may bring about ischemia, tissue anoxia, acidosis and necrosis of the involved area. In the normal case, following this defense mechanism the body attempts to affect repair by fibroblastic proliferation and new capillary formation into the affected area. This reparative process necessitates an increased oxygen uptake in the involved tissues. If the conditions that initiated the inflammatory response continue and/or there is insufficient tissue oxygen for repair to take place, a chronic inflammatory condition may ensue which, in the case of periodontal tissue, results in many of the pathological characteristics associated with periodontal disease, i.e. soft tissue and bone destruction. Thus, any process that blocks or inhibits periodontal blood flow, and thus oxygen availability, may play a role in periodontal pathology (Manhold and Weisinger, 1971).

The connection between stress and oral pathology has been well established in both human and animal studies (Manhold and Manhold, 1949; Goldberg et al., 1956; Miller et al., 1956; Moulton et al., 1952; Pierce, 1960; Giddon et al., 1962; Baker et al., 1961; Belting and Gupta, 1960; Manhold, 1958; Fedi, 1958; Gupta et al., 1960; Steinman, 1960; Hollomond, 1962; Steinman et al., 1961; Liu and Liu, 1969). It is the mechanism that

has remained somewhat elusive. Manhold, (1956), was among the first to propose a connection between the autonomic nervous system and periodontal pathology. He hypothesized a model in which increased sympathetic activity could result in a constriction of blood vessels and thus, a lack of oxygen and nutrients to the periodontium (Manhold and Weisinger, 1971; Manhold, 1956). This would be especially important during the initial reparative stages following an inflammatory episode and could lead to a pathological periodontal breakdown (Manhold and Weisinger, 1971) via a progressive, chronic inflammatory response which is unable to resolve itself. Thus, the relationship between oxygen utilization and oral health is an important one (Glickman et al., 1949). As the tissue progresses from a healthy state to a diseased state, the oxygen utilization has been experimentally shown to change. A chronic marginal gingivitis is associated with an increased O_2 utilization brought about by the formation of new blood vessels and connective tissue (Glickman et al., 1949) as the inflamed tissue attempts to heal itself. It is at this point that a restriction of oxygen and nutrients could lead to a progressive chronic inflammation and periodontal pathology. A restriction of O_2 would also favor colonization by the anaerobic bacteria associated with periodontal pathology (Grant et al., 1979b). A direct sympathetic activity in the oral cavity may provide an explanation for this pattern of progression and might help explain why people with similar oral hygiene characteristics have varying degrees of periodontal disease. Degenerating tissue (Glickman et al., 1949) and the tissue of animals subjected to chronic stress (Manhold and Weisinger, 1971) show a marked decrease in gingival oxygen utilization. A lack of oxygen during this crucial initial stage of

repair brought about by an unbalanced autonomic activity may play a role in the spread and progression of the inflammatory response.

It is also possible that autonomic induced alterations in the immune system may play a role. The immune system has also been implicated in many aspects of periodontal disease (Grant et al., 1979a, Nisengard, 1977). Although a link between these immune abnormalities and the autonomic nervous system has not been firmly established, there has been a connection made between autonomic activity and periodontal disease (Davis and Jenkins, 1962; Manhold and Weisinger, 1971). As the condition progresses the number of B-cells increase relative to T-cells (Grant et al., 1979a). As has been pointed out earlier, these B-cells are capable of producing lymphokines whose physiochemical and/or biologic activities are similar to those derived from T-cells such as migration inhibitory factor (MIF) (Yoshida et al., 1973) and osteoclast activating factor (OAF) (Mundy et al., 1974). Although it has not been experimentally demonstrated, it is reasonable to assume that the lymphocytes involved in periodontal pathology are also capable of releasing ACTH-like and interferon-like lymphokines, which are capable of acting both locally and peripherally. In order to examine the connection between autonomic activity, immune characteristics and oral disease, an adequate model should be used. The oral characteristics of the SHR which possesses an inherent sympathetic overactivity and resultant immune abnormalities have never been examined.

Very little work has been done to suggest a common etiology of hypertension and periodontal disease. Although much is known concerning the relationship between stress and the characteristics of advanced hypertension, it is only relatively recently that any focus has

been given to an autonomic-immune component (Norman and Dzielak, 1986). The same can be said for periodontal disease. Here, the main focus has been in the area of microbiology, bacteriology and their relationship to the advanced stages of the disease. A relatively small emphasis has been given to those peripheral factors which allowed the bacterial changes and proliferation to occur in the first place. Once again, although a connection has been shown between stress and oral disease (Davis and Jenkins, 1962; DeMarco, 1976; Manhold and Weisinger, 1971; Shields, 1977), it is only quite recently that an autonomic-immune component to the etiology has been considered. Current treatment modalities for both hypertension and periodontal disease are often aimed at the end results of the disease process rather than the initial mechanism. By examining the characteristics of both disease processes, a common etiology and hence, better understanding, can be accomplished.

A possible simplified mechanism of action may be initiated by a chronic stress (bacterial invasion, social stress, emotional and physiological disharmony) situation. In a chronic stress situation, an adaptation response will occur for cortisol and the increased levels will eventually diminish. This is not the case with catecholamines. Upon repeated stresses, the catecholamine levels will still increase (Rose, 1980). This stress can induce activation of the sympathetic nervous system (SNS) to the oral cavity as well as peripheral immune organs. This decreases the number of T-cells, especially T-suppressor cells (Norman and Dzielak, 1986), relative to B-cells and thus, removes control of B-cell and remaining T-cell lymphokine secretion provided by the T-suppressor cells. The unchecked B-cells and remaining T-cells are then able to increase secretion

of lymphokines such as ACTH-like substances, interferon-like substances, as well as others (OAF, MIF, etc.) which can act locally in the mouth as well as directly upon the hypothalamus and other structures to further potentiate ANS activity. During certain stress situations, it may be lymphocyte derived ACTH rather than pituitary derived ACTH that mediates increased cortisol secretion (Blalock, 1984; Blalock et al., 1982).

CHAPTER 2
A METHOD OF MEASURING THE IMMUNE SYSTEM IN RODENTS

Introduction

Over the past several years there has been an explosion of studies in the field of psychoneuroimmunology. This field attempts to examine how the brain and behavior can influence health and the susceptiblilty to disease through the immune system. Lymphocytes are a major effector cell in the immune system. There are two major types, T-lymphocytes and B-lymphocytes. T-cells are lymphocytes which develop in the thymus. The thymus is seeded during embryonic development by stem cells from the bone marrow (Male, 1986a). There is a considerable amount of T-cell proliferation and death in the thymus. Most lymphocytes will die before ever leaving the thymus. During their development in the thymus, T-cells acquire their antigen receptors and differentiate into distinct subpopulations. One of these subpopulations is the T-nonhelper cells. This population includes the T-cytotoxic cells which are capable of destroying altered "self" cells and also virally infected "self" cells. The T-nonhelper population also includes the T-suppressor cells which regulate the action of other T-cells and B-cells. Their suppressive action can be either specific for cells with particular antigen receptors or non-specific (Male, 1986a). Another distinct population of T-cells are the T-helper cells. These cells release lymphokines which can activate macrophages and other T-cells as well as help B-cells to produce antibody (Male, 1986a). B-

cells are lymphocytes which develop in the bone marrow. B-cells that are stimulated by lymphokines released by T-cells, differentiate into plasma cells that are antibody producing cells. These cell to cell interactions are summarized in Figure 2-1. The chemical mediators released by all these different cell types can act locally at tissue sites of immune system activity and directly or indirectly feed back on the CNS.

There are several different ways of measuring the activity of the immune system, natural killer (NK) cell activity, spleen cell "panning" (Wysocki and Sato, 1978), thymus weights, IgG levels, but to measure the immune status accurately, the blood concentrations of lymphocytes and proportions of B-cells to T-cells needs to be measured. The method described here was developed specifically for blood lymphocyte measurement in the rat. The percentages of T-cells, T-nonhelper cells, T-helper and B-cells are given by this method. It is accurate and rapid.

Methods

Blood Sampling

The rats are anesthetized with Metofane anesthesia (Pitman-Moore, Inc., Washington Crossing, NJ). The area of skin covering the left femoral vein was prepared and cleaned with a 70% ethanol solution. A two centimeter incision was made and the femoral vein exposed using minor dissection. Using a 25 gauge, butterfly scalp vein infusion set (Abbott Hospitals, Inc., North Chicago, IL) venipuncture was performed and one milliliter (ml) of blood obtained. A cotton swab was used to apply direct pressure to the vessel upon removal of the needle. Wound clips were used to close the incision and the blood was placed in sterile vacutainer tubes (Becton-Dickinson, Rutherford, NJ). This method is a fast and

efficient mode of blood collection as well as venous drug delivery. It is also useful when repeated samples are needed from the same animal over time, as the same incision and vessel can be used. With practice, blood samples can be collected from seven to ten animals in an hour. Also, this method has many advantages over venous catheterization.

Lymphocyte Preparation

The one ml whole blood sample was combined with one ml of PBS with 0.1% NaN_3 (pH = 7.4) that had been chilled on ice. This solution was layered on top of three ml of Lymphocyte Separation Medium (LSM, Organon-Teknika, Durham, NC) in a 14 ml conical tube that had been lightly coated with fetal bovine serum (FBS). The tubes were centrifuged at 400G for 30 minutes at 12°C. Following centrifugation, the white layer found between the plasma and LSM is predominantly composed of lymphocytes. This layer was aspirated and placed in a tube coated with FBS and containing one ml of chilled PBS with 0.1% NaN_3. All subsequent tubes were lightly coated with FBS to prevent adhesion of cells to the surface of the plastic tubes. The lymphocyte suspension was washed twice with chilled PBS with 0.1% NaN_3 prior to staining with monoclonal antibodies. The cells are washed by adding the PBS and centrifugating at 400g for 5 minutes. This is followed by aspiration of most of the supernatant, vortexing the pellet and the addition of the next 3 ml of chilled PBS.

Four lymphocyte populations were examined: T-cells, T-nonhelper cells, T-helper cells and B-cells. Mouse anti-rat monoclonal antibodies (Accurate Chemical, Westbury, NY) were used as primary antibodies and a goat anti-mouse FITC conjugated reagent (Accurate Chemical) was used as a secondary antibody. As a control, mouse IgG was used.

The specificity of each monoclonal antibody was provided by the company as follows: Clone W3/13 HLK (T-cells) labels all thymocytes and peripheral T-lymphocytes, but not B-lymphocytes; antigen also in brain. Clone OX8 (T-nonhelper cells) labels most thymocytes and the peripheral T-cell subset which includes cytotoxic and suppressor T-cells. The T-helper subset is not labelled. Some natural killer cells are also labelled. Clone W3/25 (T-helper cells) labels most thymocytes and the helper T-lymphocyte subset. No other cells are known to be recognized. Clone OX33 (B-cells) recognizes that portion of the rat leucocyte common antigen seen on B-lymphocytes. The secondary antibody used was a fluorescein conjugated goat anti-mouse IgG produced to have minimal cross reaction to human, bovine, horse and rat serum proteins.

For each sample approximately 1.5 x 10^6 cells in a 300 microliter (ul) volume were placed in each of five, labelled, 12 x 75, clear plastic tubes. The tubes were labelled as follows: control, T-cell, T-nonhelper, T-helper and B-cell. The appropriate concentration of mouse IgG or monoclonal antibody was added to each tube and the tubes were incubated for 30 minutes at four degrees centigrade. Our lab has obtained good results by adding three ul of the stock antibody solution (the ascites fluid form) to the 300 ul lymphocyte suspension. Following this initial incubation, the cells were washed three times with three ml of PBS with 0.1% NaN3.

After the third wash, the tubes were aspirated to approximately 300 ul and then 100 ul of a 1:100 dilution of the secondary FITC labelled antibody was placed in each tube. This should be done in a room with the overhead lights turned off and considerable care should be exercised in the remaining steps to protect the samples from as much light as possible.

The cells were again incubated for 30 minutes at four degrees centigrade. After an additional three washes with three ml of PBS with 0.1% NaN3, each tube was adjusted to a final volume of approximately 500 ul prior to fluorescence activated cell sorting (FACS) analysis. A summary of these steps in shown in Figure 2-2.

Results

Fluorescence activated cell sorting analysis involves hitting a thin stream of the cell suspension with a laser and then utilizing light detectors to collect the scattered light. All samples were run on a FACStar Plus (Becton-Dickinson, Rutherford, NJ). Information regarding cell size and granularity can be obtained and utilized to selectively study a single population of cells, such as lymphocytes in this case (Figure 2-3). Side scatter is an indication of cell granularity while forward scatter tends to be an indication of cell size. As is seen in Figure 2-4, fluorescently labelled cells can also be quantified by this method. Background fluorescence can be deleted and the percentage of labelled cells quantified.

We have shown here a method for identifying and quantifying different lymphocyte populations in rats. The method is quick, efficient and reliable. It is also highly reproducible. This method provides another tool for looking at the immune system during a variety of experimental conditions.

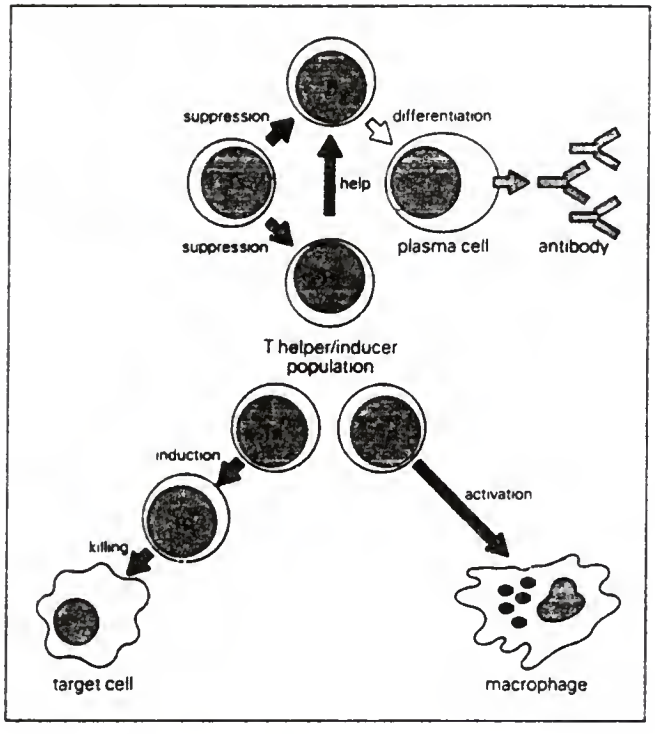

Figure 2-1: The relations and interactions of lymphocytes. From: Male D (1986a): Immunology. An illustrated outline. The C.V. Mosby Co., St. Louis, pg. 4

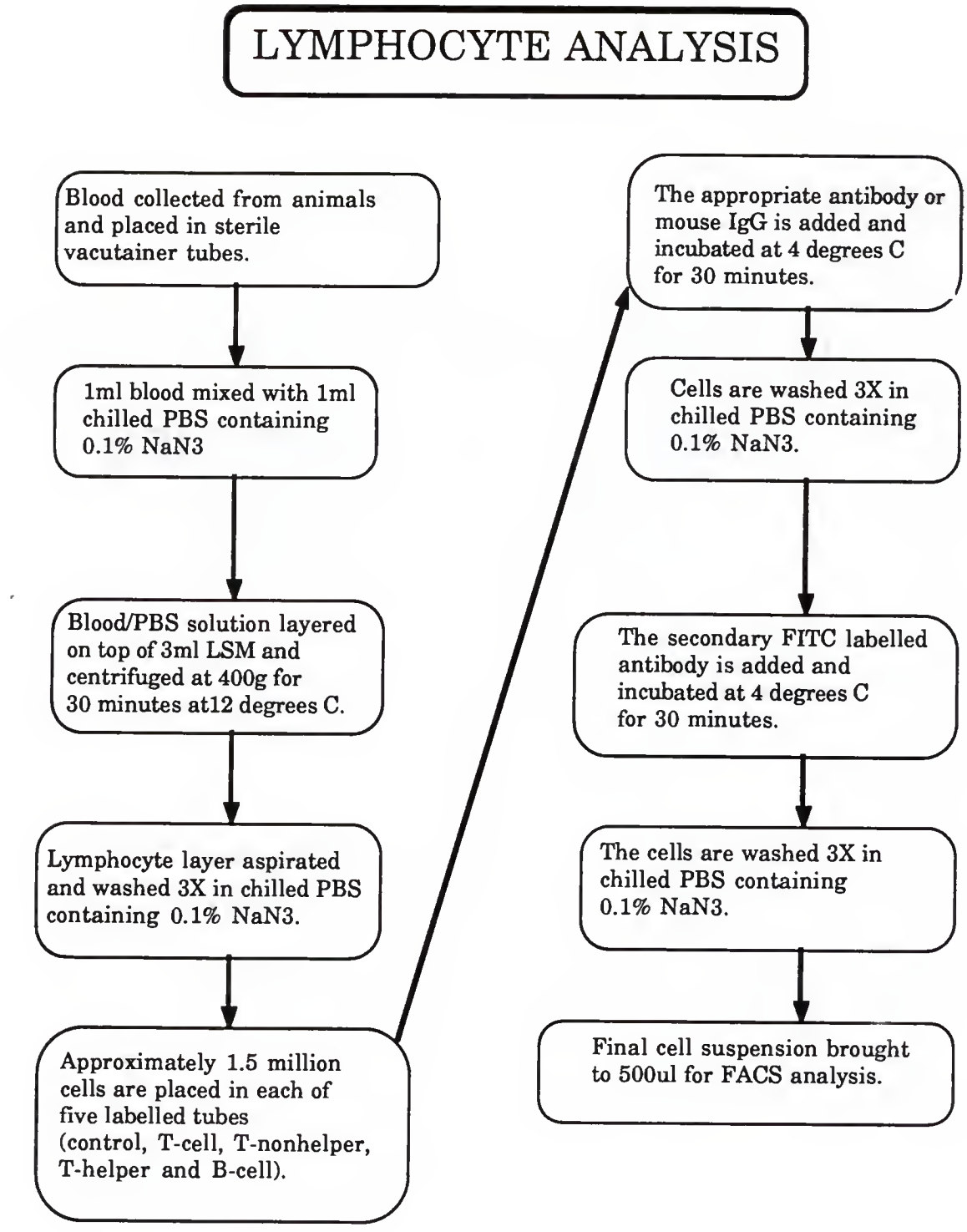

Figure 2-2: Summary of lymphocyte prepartation steps.

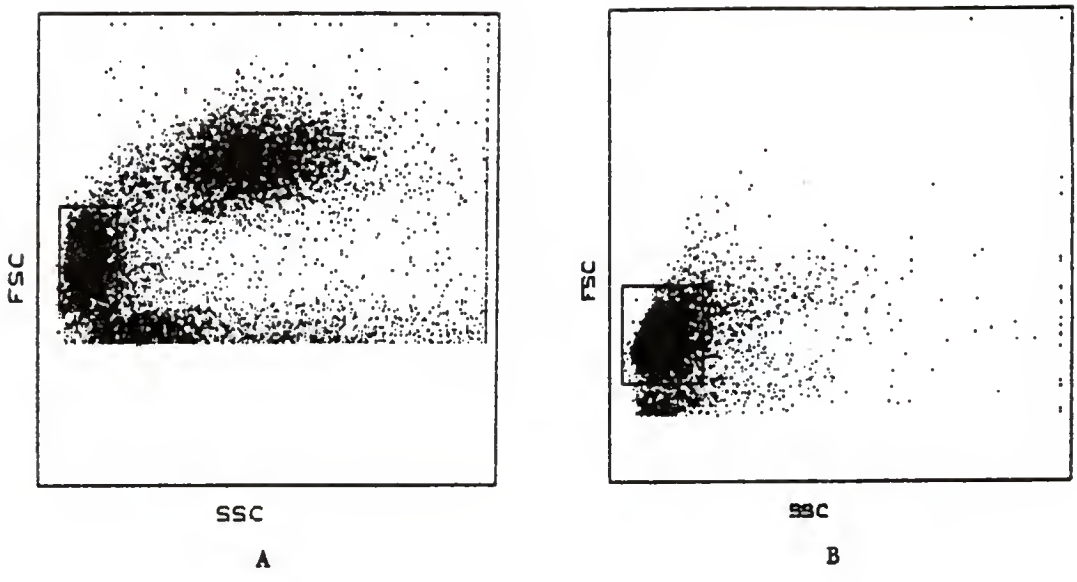

Figure 2-3: Fluorescence activated cell sorting of rat blood. Side scatter is an indication of cell granularity, while forward scatter tends ot be an indication of cell size. A: Whole blood that has had red blood cells lysed. Populations of lymphocytes, monocytes and macrophages can be seen. B: Whole blood that was separated using Lymphocyte Separation Medium. Note the relative purity of the lymphocyte population. In both cases the lymphocyte population has been boxed in.

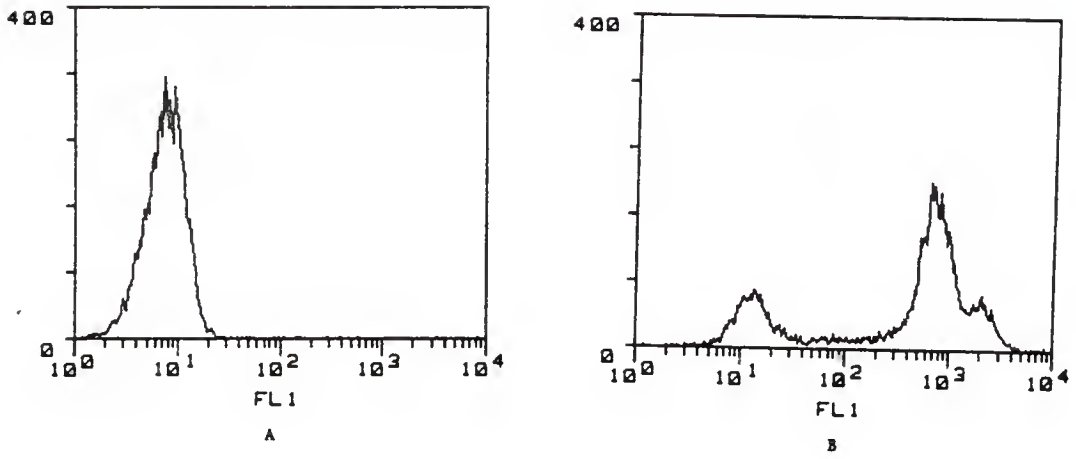

Figure 2-4: Fluorescence activated cell sorting analysis of rat blood. A: Background fluorescence in unstained cells. B: Cells that have been fluorescently labelled to identify T-cells. Background fluorescence can be deleted and the percentage of labelled cells quantified.

CHAPTER 3
CHRONIC ICV INFUSION OF NEUROPEPTIDES ALTERS LYMPHOCYTE POPULATIONS IN EXPERIMENTAL RODENTS

Introduction

A cause and effect relationship between emotional state and susceptibility to disease has long been suspected. From this cause and effect relationship the emphasis has now shifted to the examination of possible mechanisms that are involved. Peptides in the brain are being investigated for their role in controlling the immune system. Corticotropin releasing factor (CRF), arginine vasopressin (AVP), Substance P (Sub P), neurotensin, vasoactive intestinal polypeptide (VIP), somatostatin and opiod peptides are examples of neuropeptides with reported immunomodulatory properties (Yirmiya et al., 1989; Morley and Kay, 1986). Corticotropin releasing factor is a peptide linked to neural responses of stress; central administration of CRF has been studied in relation to its effects on the sympathetic nervous system (SNS) (Brown et al., 1982) and the immune system (Irwin et al., 1988). It appears that the effect of CRF is to decrease the natural killer cell activity. This has been shown to be through an action on the SNS (Irwin et al., 1988). Several studies have looked at the SNS and its influence on the immune system (Ader, 1981; Braun et al., 1985). The general hypothesis is that stressors induce elevation of the autonomic sympathetic nervous system which compromises the immune system and increases the chance of

opportunistic infection or reduced antitumor resistance. Angiotensin II and substance P are two other important neuropeptides that have shown the ability to increase SNS activity. These peptides may act in the same way as CRF to alter different lymphocyte populations.

When delivered into the lateral brain ventricles, Ang II has been shown to produce an increase in sympathetic output, as indicated by an increase in plasma norepinephrine levels (Unger et al., 1981). Other phenomena associated with intracerebroventricular (ICV) Ang II infusion include an increase in blood pressure and drinking as well as an increase in AVP secretion (Phillips, 1987). Administration of Ang II to the brain has also been associated with the release of ACTH (Reid and Day, 1977; Maran and Yates, 1977). Sub P also has been shown to cause an increase in SNS activity but does not show the increase in AVP secretion characteristic of central Ang II infusion (Unger et al., 1981). The effect of centrally administered Sub P on ACTH release is unknown. When injected centrally, Sub P seems to be more of a pure SNS activator .

In the present study we chronically infused Ang II and Sub P into the brains of intact rodents for one month and two weeks respectively. Following this chronic administration, several populations of lymphocytes were examined in order to further understand the effect of these centrally active peptides on the immune system.

Purpose

This study was to examine the effect of peptides that increase SNS activity on the percentage of the different lymphocyte populations that were of interest.

Rationale

The sympathetic nervous system has been shown to influence the immune system. Specifically, an increase in SNS activity is associated with a decrease in immune activity.

Methods

Animals

Four to five month old, male, Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Upon arrival all animals were placed in a room with a 12:12 light/dark cycle with ad lib rat chow (Purina Mills, St. Louis, MO). Water intake was measured using tap water in graduated drinking flasks.

Implantation of Osmotic Minipumps

Angiotensin II (Sigma Chemical, St. Louis, MO) was to be delivered into the lateral brain ventricle for a chronic infusion of one month via osmotic minipumps. In preparation for surgery, the animals were anesthetized with 5% chloral hydrate in saline (0.8 ml per 100g body weight). A shaved area of skin over the skull to the middle of the back was washed with a betadine solution, followed by 70% ethyl alcohol. Two incisions were made; intrascapularly and on the skull. The tissue covering the skull was pushed away until the bregma could be identified.

A 22 gauge, L-shaped cannula (Small Parts, Inc., Miami, FL) was attached to the filled osmotic minipumps via non-kinking vinyl tubing (Bolab, Inc., Lake Havasu, AZ). The cannula was fed under the skin from the intrascapular incision to the skull incision. The cannula was then stereotaxically placed at L=1.0mm, P=1.0mm, V=-5.0mm with respect to

Bregma. Methyl methacrylate cement was used to attach the cannulae to previously placed retention screws.

Alzet osmotic minipumps (Alza Corp., Palo Alto, CA) were used for drug delivery. The model 2002 pumps deliver at a rate of 0.5 µl per hour for 14 days. A sterile Ang II solution was prepared to a concentration that would allow for a one ug/hr release of Ang II over the 14 day life of the pump. The Ang II was dissolved in a vehicle of artificial cerebrospinal fluid (CSF), the formula for which was supplied by the Alza Corporation.

Angiotensin II has been used in several studies involving Alzet osmotic minipumps. We had some question, however, regarding the stability of the Ang II over the entire functional life of the pump. We placed several model 2002 pumps in flasks containing normal saline and then placed these flasks in a water bath at 37°C for 14 days. All pumps contained Ang II dissolved in artificial CSF. Using radioimmunoassay, we found that immunoreactive Ang II was being delivered by the pumps at the rate stated by the company.

As the model 2002 pump is only used for a 14 day period, the Ang II containing pumps were replaced on day 14. Metofane anesthesia (Pitman-Moore, Inc., Washington Crossing, NJ) was used for this brief procedure.

Substance P was to be delivered into the lateral brain ventricle for a chronic infusion of two weeks. The procedure was the same as that for Ang II except that the Sub P was dissolved in the artificial CSF to a concentration that would allow for a two ug/hr release of Sub P over the 14 day life of the pump.

Drinking Measurements

Only animals that showed an increase in drinking, relative to controls, were utilized as Ang II infused animals.

Blood Pressure Measurements

Systolic blood pressure was measured in the Sub P rats on day 12 using a tail plethysmograph.

Lymphocyte Preparation

One milliter of whole blood was obtained from the left femoral vein under metofane anesthesia as described in Chapter 2. Lymphocytes were separated and prepared for FACS analysis as described in Chapter 2.

Statistics

Student's t test was used to compare the means of the two groups for each experimental protocol (Ang II infusion or Sub P infusion). A significance level of 0.05 was used.

Results

Angiotensin II is a powerful dipsogen when injected directly into the brain ventricles. The effect is so reliable that a measurement of drinking is a valid measure of cannula patency in the brain ventricles. Fig. 3-1 shows that the constant infusion of Ang II via the minipumps was effective as illustrated by the increase in drinking by the experimental group.
Figure 3-2 shows graphically the results of quantifying the percentage of different lymphocyte populations. As can be seen, there was a significant increase in the percentage of T-cells (84.5±3.1 vs 74.1±2.3) and a decrease in the percentage of B-cells (14.2±1.6 vs 20.3±0.9), present in the Ang II infused rats, relative to the artificial CSF infused controls. In the Sub P infused rats, there was also a significant increase in the percentage of T-cells relative to the control animals (83.8±3.4 vs 74.3±1.4) (Figure 3-3). The

Sub P infused animals, however, did not show the decrease in the percentage of B-cells that was seen in the Ang II animals. No significant differences were noted in the T-nonhelper or T-helper populations of either experimental group.

Acute Sub P injections into the brain ventricles have been shown to increase blood pressure (Unger et al., 1981). Although the Sub P infused animals showed a trend toward higher blood pressure after 12 days relative to the artificial CSF control animals, the groups were not statistically different (Table 3-1).

Discussion

Our goal was to look at two experimental models of centrally induced increased SNS activity and see if this resulted in any alteration in the percentage of different lymphocyte populations. Other measures of the immune system have been used to show that CRF alters immune competence (Irwin et al., 1988). This effect is due to sympathetic action on the immune system. Centrally infused Ang II has been shown to cause an increase in sympathetic nervous system activity (Unger et al., 1981; Phillips, 1987) as has Sub P (Unger et al., 1981). Therefore, these changes are most likely due in part to this increased SNS activity.

It is interesting to speculate on why the Ang II infused animals showed a decrease in B-cells and why the Sub P animals did not. As has been mentioned previously, Ang II infused into the brains of intact rodents causes not only an increase in SNS activity, but also an increase in AVP release (Unger et al., 1981; Phillips, 1987) and ACTH release (Reid and Day, 1977; Maran and Yates, 1977). AVP has been suggested to play a role in immunoregulation (Yirmiya et al., 1989). AVP immunoreactivity has been

found in such tissues as lymph nodes (Aravich et al., 1987) and thymus (Markwick et al, 1986). AVP receptors are found on lymphocytes and these receptors are thought to play a role in lymphokine production (Johnson and Torres, 1985). The possibility exists that AVP may be acting on certain lymphocyte populations, such as the T-helper population, to influence the production of B-cell sensitive lymphokines. ACTH also has immunoregulatory properties through its action on corticosteroid release. Macrophages are particularly sensitive as corticosteroids can inhibit their activation. Lymphocytes are also effected. Corticosteroids inhibit the primary antibody response and reduce the numbers of circulating T-cells, especially T-helper cells (Male, 1986b). The data from this experiment, however, do not indicate a reduction in any T-cell population examined. In fact, an increase in the percentage of total T-cells was observed.

Also of interest is the comparison of this work with experiments done in our lab on genetic models of increased sympathetic nervous system activity. The spontaneously hypertensive rat (SHR) has been shown to have an increased SNS activity that is at least in part responsible for the hypertensive state (Norman and Dzielak, 1986; Judy et al., 1976). We have shown that adult SHR demonstrate a decrease in the percentage of lymphocytes from each of the populations studied (Chapter 4). Although both the genetic and experimental models have an increased SNS activity, clearly other factors are at work here.

By changing the B-cell and T-cell ratios, many aspects of the immune response are altered affecting a variety of body functions. SHR exhibit increased brain levels of Ang II (Phillips and Kimura, 1988) and increased sympathetic nerve activity (Norman and Dzielak, 1986; Judy et al., 1976).

Several investigators have suggested changes in the immune system as at least a partial cause of the hypertensive state (Takeichi et al., 1986; Khraibe et al., 1984; Takeichi et al., 1981; Fernandes et al., 1986) in this model. These changes involve alterations in the B-cell, T-cell ratio which appears to be significant in the pathogenesis of hypertension (Khraibe et al., 1984; Bendich et al., 1981). Bulloch (1985) has shown that the ANS sends fibers to the immune organs. These fibers richly innervate areas high in T-cell concentration and avoid areas that contain developing B-cells.

Based upon this information a possible simplified mechanism of action may be hypothesized. In a chronic stress (bacterial invasion, social stress, emotional and physiological disharmony) situation, an adaption response will occur for cortisol and the increased levels will eventually diminish. This is not the case with catecholamines. Upon repeated stresses, the catecholamine levels will still increase (Rose, 1980). This stress can induce activation of the sympathetic nervous system (SNS) to various parts of the body, including peripheral immune organs. This decreases the number of T-cells, especially T-suppressor cells, relative to B-cells and thus, removes control of B-cell and remaining T-cell lymphokine secretion provided by the T-suppressor cells. The unchecked B-cells and remaining T-cells are then able to increase secretion of lymphokines such as ACTH-like substances, IFN-like substances, as well as others (OAF, MIF, etc.) which can act locally in tissues, as well as directly upon the hypothalamus and other structures to further potentiate ANS activity. It is the B-cell to T-cell ratio that is important. This view is supported by the fact that during certain stress situations, it may be lymphocyte derived ACTH rather than pituitary derived ACTH that mediates increased cortisol secretion (Blalock et al., 1982; Blalock, 1984).

In summary, we chronically infused neuropeptides into the brains of intact rodents. We found alterations in the percentages of different lymphocyte populations. Specifically, the Ang II infused animals demonstrated a decrease in the percentage of B-cells and an increase in the number of T-cells. The Sub P infused animals also exhibited this increase in T-cells, but failed to show any alteration in the percentage of B-cells. Finally, this study adds to the growing body of data suggesting an important role of the CNS in regulating immune function and susceptibility to disease.

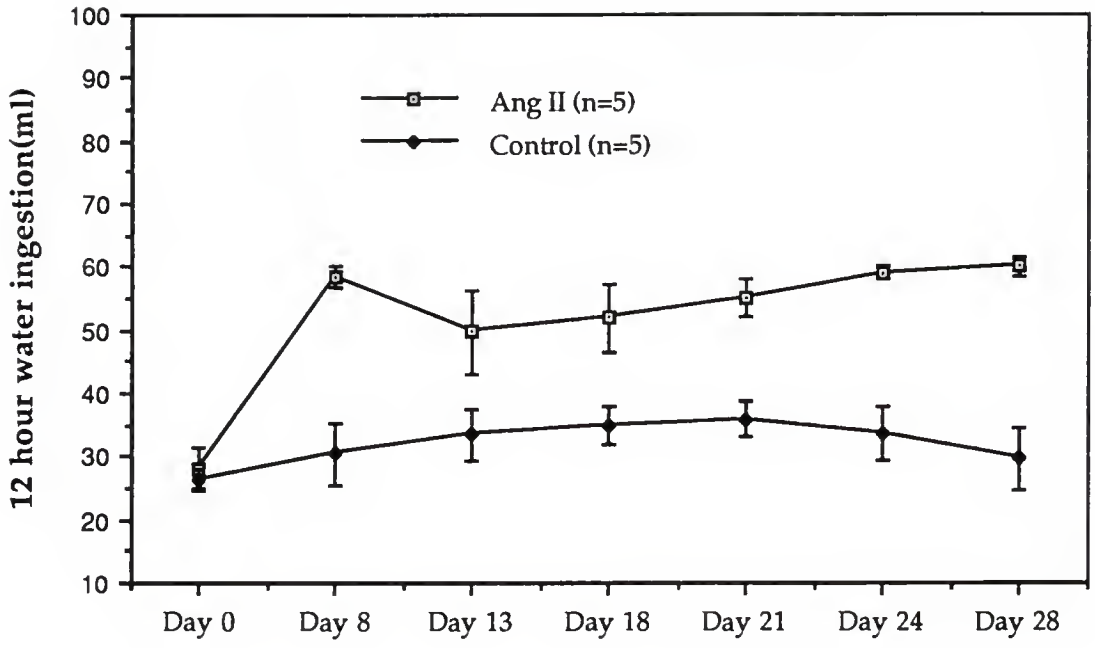

Figure 3-1: Drinking response to chronic ICV Ang II infusion via osmotic minipumps in Ang II infused rats versus artificial CSF infused control rats. Water intake was measured as total amount consumed when water bottles were available for 12 hours. All values are expressed as Mean ± SEM.

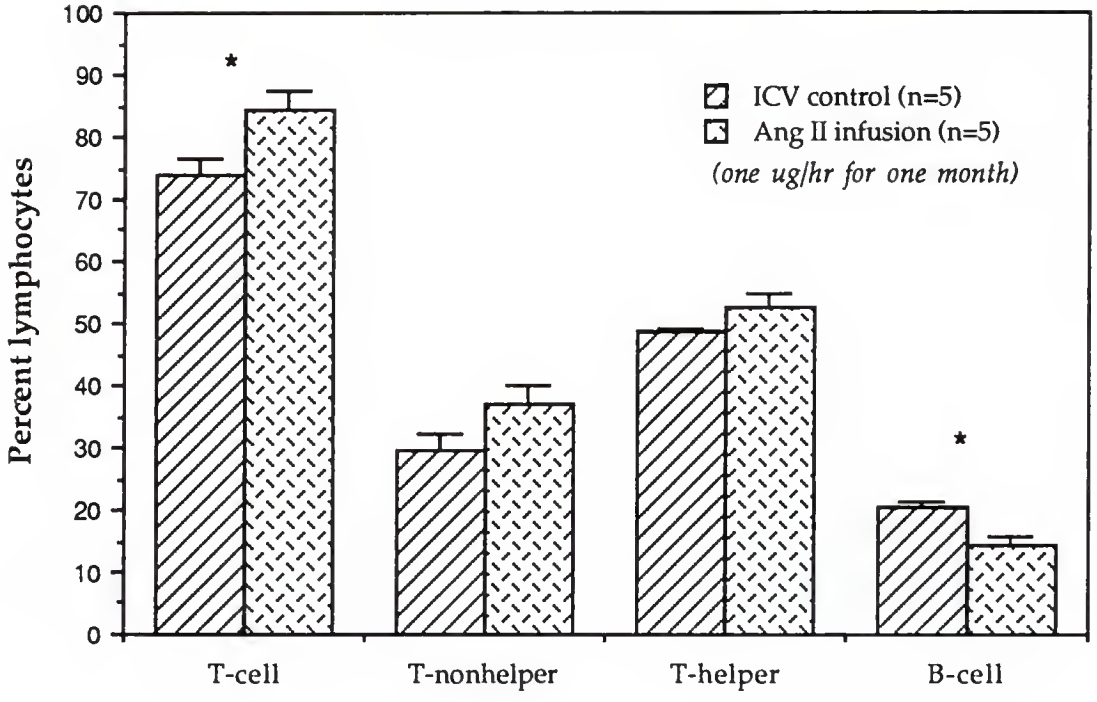

Figure 3-2: Percentage of different lymphocyte populations in Ang II infused rats versus artificial CSF controls. * = $p < 0.05$. All values are expressed as Mean ± SEM.

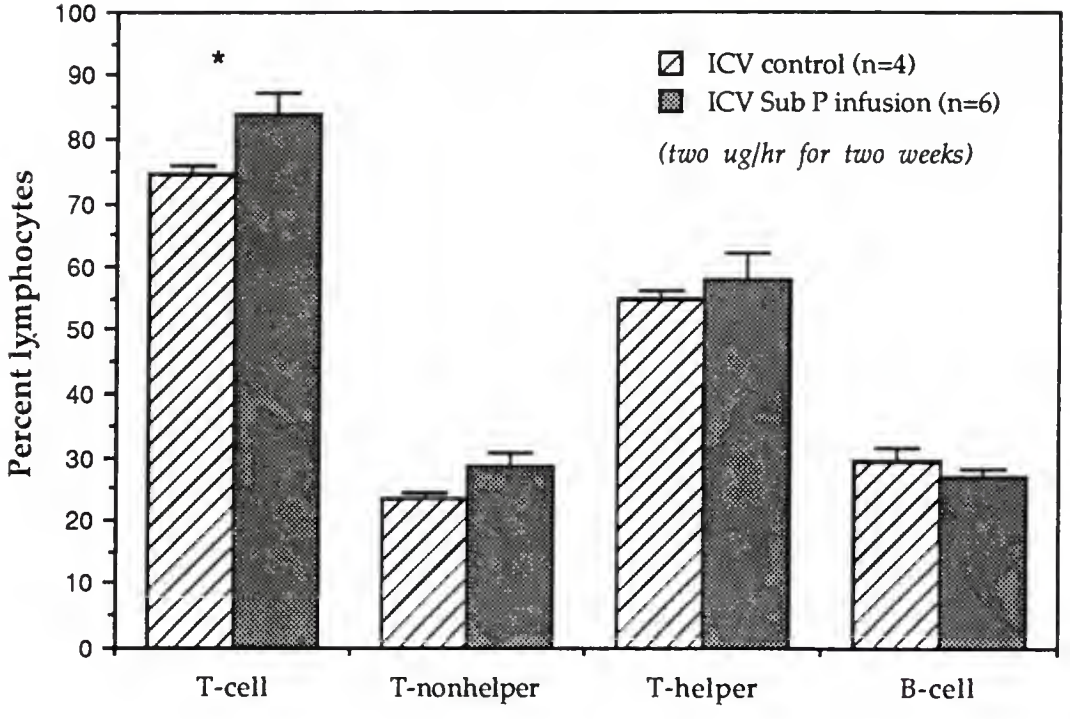

Figure 3-3: Percentage of different lymphocyte populations in Sub P infused rats versus artificial CSF controls. * = $p < 0.05$. All values are expressed as Mean ± SEM.

TABLE 3-1:
SYSTOLIC BLOOD PRESSURES OF ANIMALS INFUSED WITH SUB P FOR 12 DAYS

	N	BP
Control	4	119±9.0
Sub P	6	127±4.9

All values are Mean ± SEM.

CHAPTER 4
ALTERATIONS OF LYMPHOCYTE POPULATIONS DURING DEFVELOPMENT IN THE SPONTANEOUSLY HYPERTENSIVE RAT

Introduction

The spontaneously hypertensive rat (SHR) is widely used as one of the models for human essential hypertension. The causes of this hypertensive state are not yet fully understood and many possibilities are being investigated. Among the factors considered are alterations in the central renin-angiotensin system (Phillips and Kimura, 1988), increased sympathetic nervous system (SNS) activity (Norman and Dzielak, 1986; Judy et al., 1976) and increased arginine vasopressin (AVP) secretion (Crofton et al., 1978). Some evidence is accumulating to lend support to the idea that the immune system is also involved in the development of hypertension in the SHR (Norman et al., 1985; Norman and Dzielak, 1986; Takeichi et al., 1981; Fernandes et al., 1986; Khraibi et al., 1984). Thymic transplants from neonatal WKY to neonatal SHR will attenuate the development of hypertension (Norman et al., 1985). Also, immunosuppressive drugs, such as cyclophosphamide, will attenuate the development of hypertension in SHR (Khraibi et al., 1984) Immune system abnormalities, such as increased levels of serum immunoglobulins and the presence of antibodies directed against self vascular components, have also been noted in hypertensive human subjects (Svendson, 1979; Raff and Wortis, 1970; Mathews et al., 1974). These studies looking at

immune factors in the SHR have used adult rats that already had established hypertension (Norman and Dzielak, 1986; Takeichi et al., 1981; Fernandes et al., 1986). Therefore, the immune status could have resulted from the hypertension. The goal of this study was to look for alterations in lymphocyte populations that occur in the pre-hypertensive as well as during the hypertensive stages of development of these animals.

Lymphocytes are a major effector cell in the immune system. Besides being involved in antibody production and direct cell killing, activated lymphocytes secrete lymphokines, which include various chemical mediators that are capable of acting locally as well as centrally (Blalock, 1984; Grant et al., 1979a). Any differences in the immune system in the pre-hypertensive state will provide additional evidence linking immune system alterations to the development of hypertension. The SHR begins to develop hypertension at four to five weeks of age. Blood pressure rises rapidly for the next two to three months and reaches an asymptote at four months (Figure 4-1). Therefore, we studied rats at two weeks, four weeks, two, three and four months of age.

There are four major components of the immune system's lymphocyte populations which can be measured in the rat using specific monoclonal antibodies. These include the B-cells, the T-cells, and two subpopulations of T-cells, the T-helper cells and the T-nonhelper cells which include the cytotoxic T lymphocytes, the suppressor T lymphocytes and some natural killer (NK) cells. The purpose of this present study is to examine different lymphocyte populations at various time points during the development of high blood pressure in the SHR.

Purpose

The purpose of this study was to analyze changes in lymphocyte populations occuring in the prehypertensive as well as hypertensive phases of development in the SHR.

Rationale

The immune system, especially those factors which affect the development of lymphocytes, has been implicated in the development of hypertension in the SHR. To test if this is the result of hypertension or appears before hypertension these experiments are undertaken.

Methods

Animals

Two week old, male SH and WKY rats were obtained from breeding pairs purchased from Charles River Labs, Inc (Wilmington, MA). Male SHR and WKY rats (n=8/group) used for the remaining time points were obtained at 28 days of age from Charles River Labs, Inc. A separate group of five month old, male WKY (n=5) and Sprague-Dawley (n=6) rats was also obtained from Charles River Labs for comparison of these two normotensive strains. Upon arrival, all rats were placed on ad-lib rat chow and water diets in a temperature controlled room with 12:12, light: dark cycle.

Blood Samples

In order to obtain blood from the two week old animals a thoracotomy was performed under Metofane (Pitman-Moore, Inc., Washington Crossing, NJ) anesthesia and as much blood as possible obtained via direct cardiac puncture. Only animals that produced at least 700ul were used in this study. The small number of animals (WKY, n=3; SHR, n=4) at the

two week age point is a reflection of the difficulty involved in obtaining sufficient quantities of blood from such young animals.

At 34 days of age and at four week intervals thereafter blood was obtained from the remaining animals. The same group of SHR and WKY animals was used throughout the remainder of the study. Blood was obtained from these animals as described in Chapter 2.

Lymphocyte Preparation

Lymphocytes were separated and prepared for FACS analysis as described in Chapter 2.

Blood pressure

Systolic blood pressure recordings were made in unanesthetized, restrained animals using a tail plethysmograph.

Statistical analysis

All populations were compared using Students t test for comparison of means. A level of $p<0.05$ was considered significant.

Results

Lymphocyte populations

In the prehypertensive phase, at two weeks of age, SHR demonstrate a significant decrease in the percentage of both T-nonhelper cells (14.1±1.2 vs 18.2±0.9) and B-cells (35.8±1.8 vs 39.1±1.5) compared to WKY rats ($p<0.05$) (Figure 4-2). There were no differences in the total T-cell and T-helper cell populations. At one month of age the SHR animals showed a significantly lowered T-nonhelper cell percentage (12.6±3.0 vs 18.9±3.8) ($p<0.05$) (Figure 4-3). No difference was found in B-cells. At two months of age the T-nonhelper cells were reduced relative to the WKY controls (23.0±3.2 vs 29.9±3.2) and the T-helper population in SHR was

significantly higher (55.6±1.9 vs 51.4±2.4) (Figure 4-4). The picture at three months (Figure 4-5) is similar to the picture at one month with the T-nonhelper population continuing to be reduced in the SHR (25.5±0.61 vs 30.4±2.0). The T-helper cell difference noted at 2 months was no longer significant. At four months of age we see a much more dramatic change in the lymphocyte profile (Figure 4-6). The T-nonhelper cells continued to be significantly decreased (27.7±1.8 vs 33.4±2.5) and at this time point all lymphocyte populations examined were also significantly decreased relative to the WKY animals ($p<0.05$).

Figure 4-7 shows a comparison of four month old WKY and Sprague-Dawley animals. No significant differences were noted in any lymphocyte population.

Blood pressures

Data was obtained from the SHR and WKY rats at the termination of the experiment (Table 4-1). The SHR showed a significant increase in systolic blood pressure ($p<0.05$).

Discussion

The results show that even before hypertension develops, SHR have a deficit in T-nonhelper cells. This deficit persists throughout the development and maintenance of hypertension. The SHR showed differences in their lymphocyte profile at both 2 weeks and one month of age relative to their genetic control, the WKY. This is in the prehypertensive phase of development. Both the two week and four week old SHR showed a decrease in their T-nonhelper cell population (Figures 4-2 and 4-3). This is in agreement with other studies done on mature SHR (Norman et al., 1985; Norman and Dzielak, 1986). This T-nonhelper deficit remained throughout the study period of four months. These

results confirm the finding of Norman and Dzielak (Norman et al., 1985; Norman and Dzielak, 1986) and demonstrate that the immunologic dysfunction which they found in SHR is not a secondary adaptation to hypertension but a pre-existing condition found in the SHR but not in the WKY and Sprague- Dawley normotensive rats. Therefore the decrease in the T-nonhelper population which we have found may contribute to the etiology of hypertension in the SHR. The T-nonhelper cell population includes cytotoxic T cells, suppressor T-cells, as well as some NK cells.

A major question posed by this finding is whether the impairment in T-nonhelper cells contributes to hypertension via a direct action of the immune system on vascular components or indirectly via lymphokines and other chemical mediators released from other immunoregulatory cell types acting on the CNS. The impairment of T-nonhelper cells could permit the emergence of vascular inflammation leading to the deposition of atherosclerotic plaque, or the reduction in T-nonhelper cells could be disturbing the balance of chemical mediators produced by the other cell types. SHR do exhibit numerous localized areas of vascular inflammation throughout their cardiovascular system (Tadeichi et al., 1986).

A second question is why the T-nonhelper cells are reduced in the first place. There are data to indicate that the peptides produced in the brain can influence the system (Blalock et al., 1985). One possible method of action is that centrally synthesized peptides, such as CRF, may be acting on immunocompetent cells directly. The different cell types found in the immune system have receptors for and can synthesize centrally active substances (Blalock, 1984; Johnson and Torres, 1985; Blalock et al., 1985).

However, recently, Irwin et al (Irwin et al., 1988), have demonstrated that CRF acts on the immune system by activating the SNS.

The SHR also demonstrates alterations in the brain renin-angiotensin system. Specifically, the SHR has shown higher levels of Ang II in the brain (Phillips and Kimura, 1988). When infused ICV into the brains of intact normotensive rats, Ang II has the effect of causing an increase in SNS activity as well as a increase in AVP secretion (Unger et al., 1981). This mimics the condition in the SHR which also shows increases in both SNS activity (Norman and Dzielak, 1986; Judy et al., 1976) and AVP secretion (Crofton et al., 1978). Central Ang II also plays a role in the release of ACTH. This increase in central Ang II may explain why SHR have increased plasma levels of corticosterone (Dietz et al., 1978).

The SNS has been shown to influence the immune system (Irwin et al., 1988; del Ray et al., 1985; Braun et al., 1985). The evidence suggests that SNS activity tends to attenuate immune function (Irwin et al., 1988; del Ray et al., 1985; Braun et al., 1985). It is known that the SNS sends fibers to immune organs such as the thymus, spleen and lymph nodes (Bulloch, 1985). These fibers tend to innervate areas rich in T-cells but avoid areas where high B-cell concentrations are found. Given the fact that SHR exhibit higher SNS activity, as well as other changes in their catecholamine systems (Borkowski and Quinn, 1984; Kuchel et al., 1987), it is not surprising that we would find alterations in the percentage of different lymphocyte populations.

Besides this increase in SNS activity, the SHR also demonstrates an increase in AVP secretion (Crofton et al., 1978). There are several lines of evidence that suggest an immunoregulatory role for AVP. Plasma levels

of AVP increase following exposure of rats to bacterial endotoxin. (Kastin, 1986). Receptors for AVP are found on T-cells (Johnson and Torres, 1985) and these may be influencing lymphokine production and secretion. Evidence of AVP immunoreactivity is found in important lymphoid tissues such as lymph nodes (Aravich et al., 1987) and the thymus (Markwick, 1986). Also, vasopressin deficient rats exhibit increased natural killer cell activity (Yirmiya et al., 1989). Interestingly, these rats show a decreased amount of CRF in the brain (Krieger et al., 1977). When CRF is injected centrally this results in a decrease in natural killer cell activity (Irwin et al., 1988) that is believed to be brought about by an increase in SNS activity (Brown et al., 1982). AVP can also potentiate the release of CRF and ACTH (Gillies at al., 1982; Gonzalez-Luque et al., 1970). This would impact on the immune system through the effects of gluccocorticoids. Thus, this increase in AVP secretion found in the SHR may influence the immune system through a variety of mechanisms.

AVP levels and SNS activity are altered in the SHR. These two factors have also been shown to have some influence on the immune system. The immune system itself has been implicated in the pathogenesis of hypertension in the SHR (Takeichi et al., 1981; Takeichi et al., 1986). Immunosuppression will attenuate the development of hypertension in the model (Khraibi et al., 1984). Thymic transplants from WKY to neonatal SHR will also cause these animals to become less hypertensive (Norman and Dzielak, 1986). SHR given antithymocyte serum show a reduction in blood pressure relative to controls (Bendich et al., 1981). It appears certain that the immune system is at least in part responsible for the development of hypertension in the SHR.

In the normal situation the balance between the different lymphocyte populations is carefully controlled. As has been mentioned previously, lymphocytes are capable of producing chemical mediators. Other lymphoid cells such as macrophages also secrete chemical mediators. It is through these messengers that the various cells of the immune system interact with one another. Because the chemical mediators released by one cell type may have a profound effect on the proliferation and differentiation of another cell type, it is possible that very subtle changes in certain cell types may have large effects throughout the entire immune system. Both T and B lymphocytes are capable of lymphokine secretion (Grant et al., 1979a). Included in the lymphokine category are a variety of substances including such things as osteoclast activating factors, PMN migration inhibitory factors, and ACTH-like substances, as well as others not fully explored (Blalock, 1984; Grant et al., 1979a). These substances are capable of acting at local sites as well as acting centrally. From this it is possible to hypothesize that an imbalance in various cells of the immune system may lead to the over or under production of various lymphokines that may exert numerous effects through peripheral and central action. In this particular case a decrease in the amount of suppressor cells may be releasing the brake on the action and activity of the other cell types. The resulting release of lymphokines and/or other chemical mediators could be acting centrally to influence such things as central angiotensin II levels and sympathetic output. Interestingly, lymphocytes from both prehypertensive and hypertensive SHR have a lower intracellular pH than those from WKY (Batlle et al., 1990b). This condition may further influence the production and secretion of lymphokines from these cells.

This decrease in the amount of suppressor cells may also be influencing the production of antibodies. The release of the braking effect provided by the suppressor cells coupled with the imbalance of lymphokine secretion could increase the production of autoantibodies directed against vascular components. In human hypertensive patients antibodies of this type have been identified (Svendson, 1979). The SHR does posses numerous areas of localized vascular inflammation (Takeichi et al., 1986). In the SHR the alterations in cell populations became more numerous as the animals progressed in age.

It has become apparent recently that when comparisons are made between the SHR and WKY strains, sometimes it may be the WKY animals that are "abnormal". For this reason, we compared two normotensive strains, the WKY and Sprague-Dawley. We found no differences between these strains for any of the lymphocyte populations examined. These results indicate that it is indeed the SHR that is "abnormal".

In summary we present data showing alterations in various lymphocyte populations in both the prehypertensive and hypertensive phases of development in the SHR. These results support the hypothesis that immune dysfunction is involved in the pathogenesis of the hypertensive state in this model.

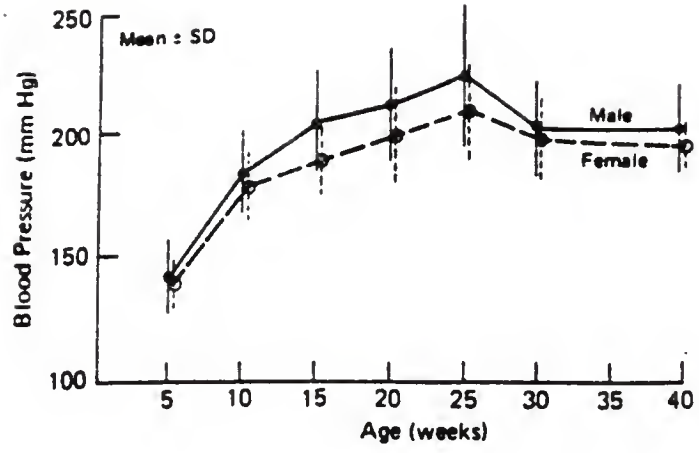

Figure 4-1 The development of hypertension in the SHR. From: Udenfriend S, et al. (1976): Spontaneously hypertensive (SHR) rats: Guidelines for breeding, care, and use. ILAR News 19(3); G7

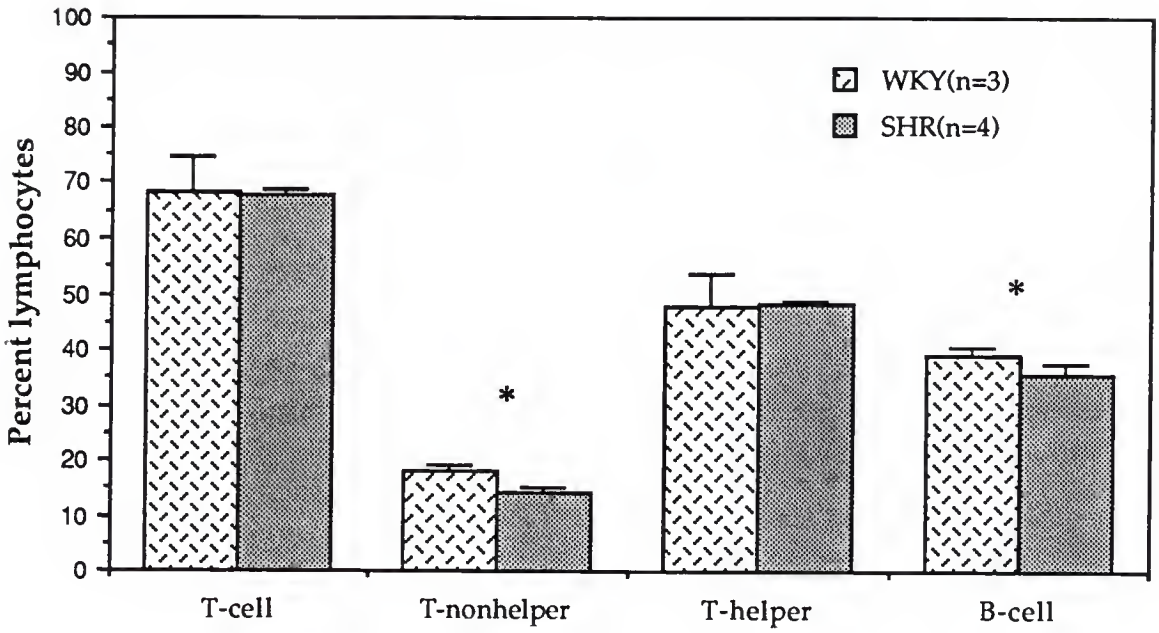

Figure 4-2: Percentage of various lymphocyte populations at 2 weeks of age in SHR and WKY animals. * = $p<0.05$ for SHR vs WKY. Values are Mean ± SEM.

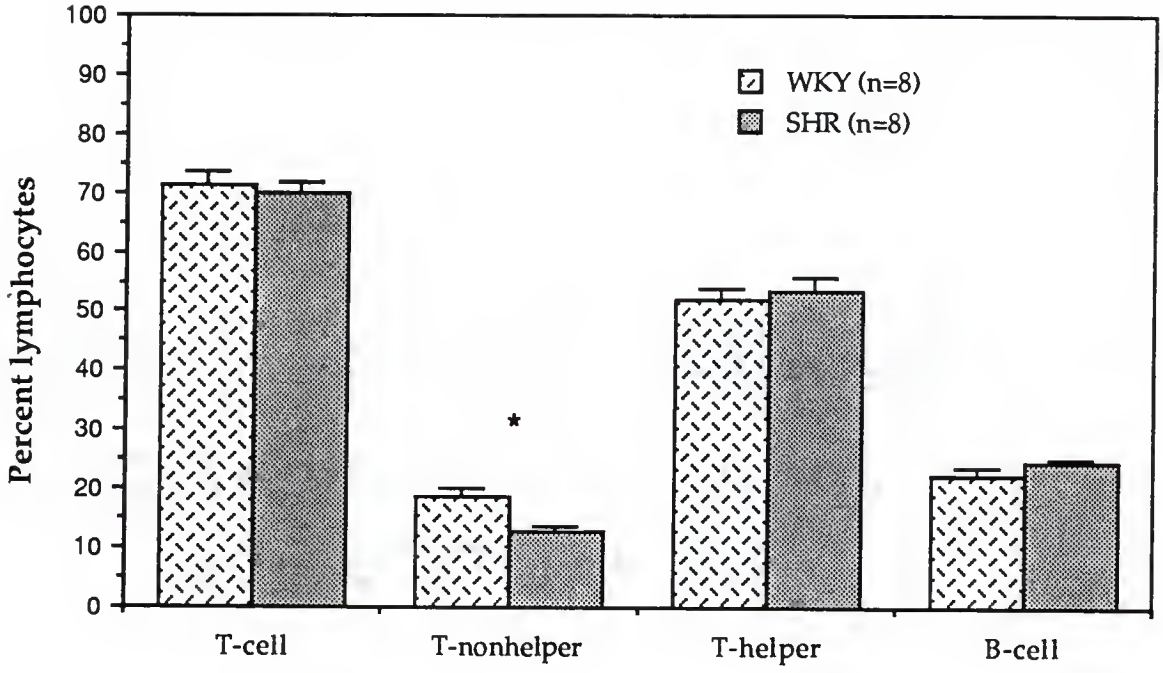

Figure 4-3: Differences in lymphocyte populations at four weeks of age in the genetically hypertensive SHR compared to its normotensive control, the WKY. * = $p<0.05$ for SHR vs WKY. Values are Mean ± S.E.M.

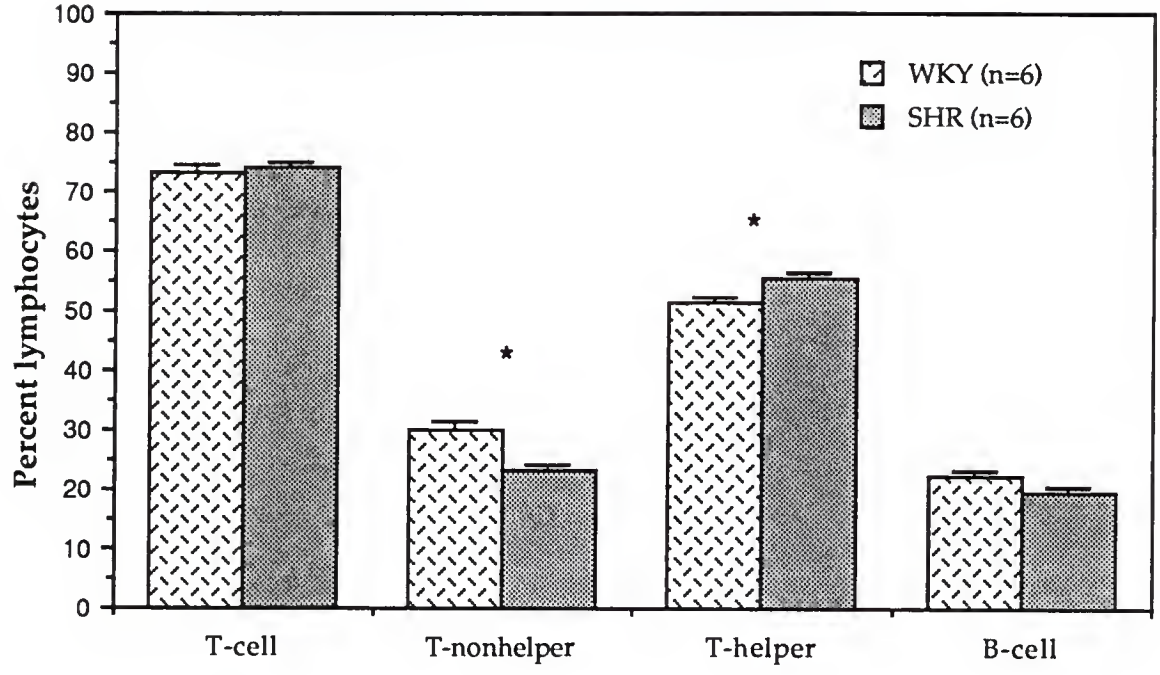

Figure 4-4: Differences in lymphocyte populations in WKY and SHR strains at two months of age. * = $p<0.05$ for SHR vs WKY. Values are Mean ± S.E.M.

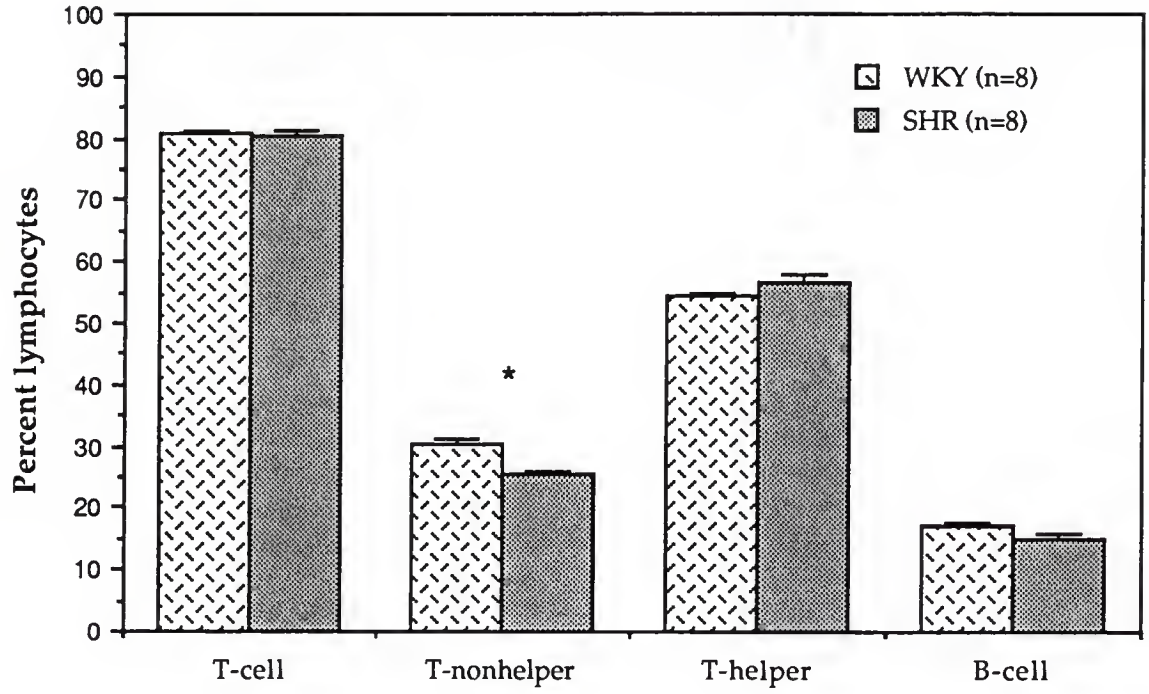

Figure 4-5: Lymphocyte population differences between the genetically hypertensive SHR and its normotensive control at three months of age. * = $p<0.05$ for SHR vs WKY. Values are Mean ± S.E.M.

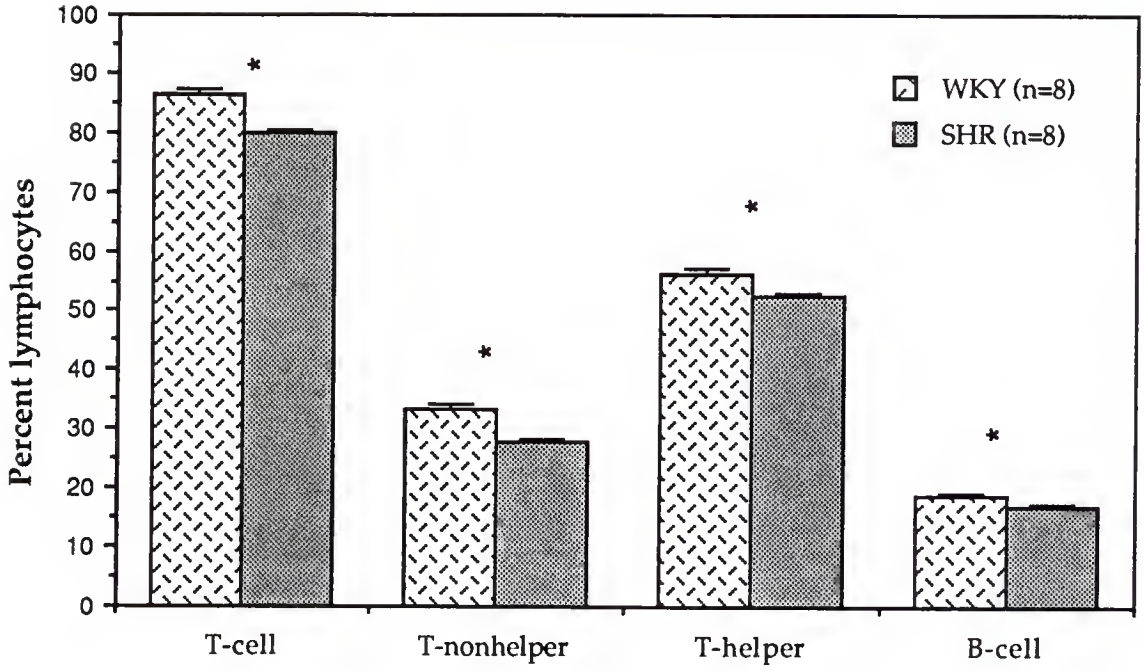

Figure 4-6: Differences in lymphocyte populations in 4 month old WKY and SHR. * = $p<0.05$. Values are Mean ± S.E.M.

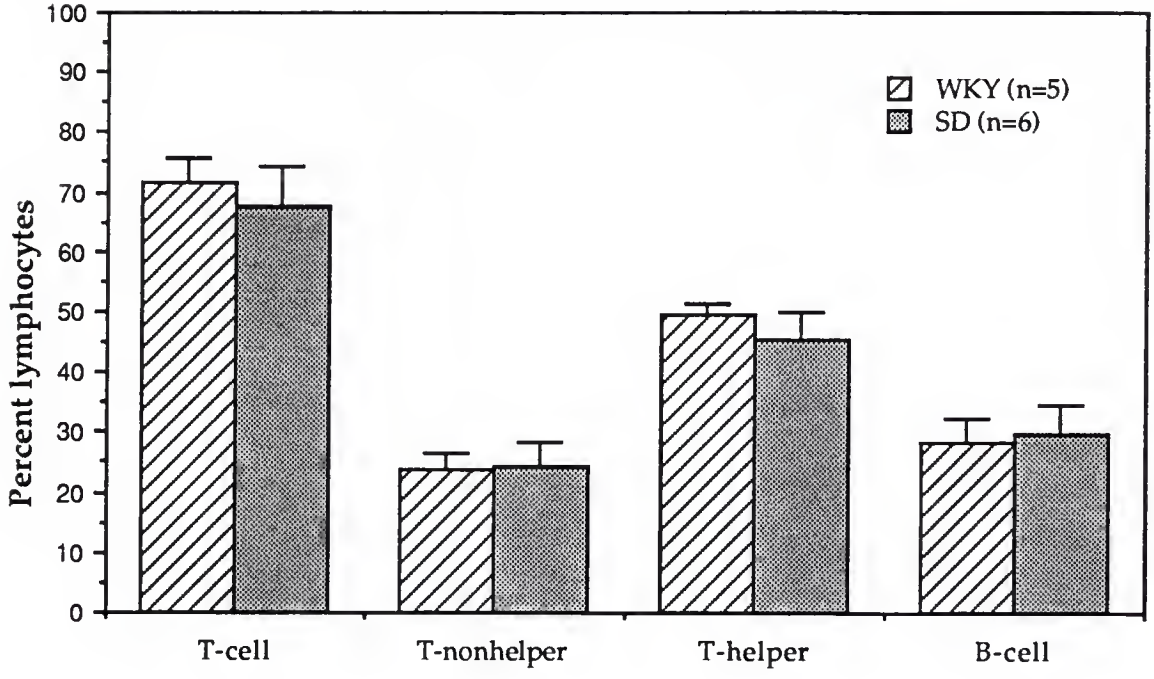

Figure 4-7: Differences in lymphocyte populations in 5 month old WKY and Sprague-Dawley (SD) rats. No significant differences were found in any lymphocyte populations. All values are Mean ± SEM.

TABLE 4-1:
SYSTOLIC BLOOD PRESSURE MEASUREMENT IN ADULT SHR AND WKY ANIMALS

	N	BP
WKY	8	128±4.5*
SHR	8	194±2.9

All values are Mean ± SEM. * = $p<0.05$ for WKY vs SHR.

CHAPTER 5
GUANETHIDINE TREATMENT ALTERS LYMPHOCYTE POPULATIONS AND REDUCES BLOOD PRESSURE IN SPONTANEOUSLY HYPERTENSIVE RATS

Introduction

Several lines of evidence point to the immune system as an important factor in the etiology of hypertension in the spontaneously hypertensive rat (SHR) (Takeichi et al., 1981; Norman et al., 1985; Bendich et al., 1981; Norman and Dzielak, 1986; Khraibi et al., 1984). Thymic implants from WKY to neonatal SHR will attenuate the development of hypertension (Norman et al., 1985; Norman and Dzielak, 1986). Treatment with immunosupressive drugs will also lower blood pressure in SHR (Khraibi et al., 1984). In our own lab, we have found alterations in the percentages of specific lymphocyte populations in SHR from as early as two weeks of age (Chapter 4). This evidence suggests that the immune system is linked to the development of hypertension in this model.

Increased sympathetic nervous system (SNS) activity is a well characterized phenomenon in SHR (Norman and Dzielak, 1986; Judy et al., 1976). This increased SNS action is one factor that has been definitely associated to the development of high blood pressure in the SHR. Increases in SNS activity have also been linked to alterations in immune system function and components, such as a decrease in natural killer cell cytotoxicity (Irwin et al., 1988). In addition, regional sympathetic denervation has been shown to stimulate local immune responses (Braun

et al., 1985). Thus there is evidence to suggest that the sympathetic nervous system influences the immune system in SHR and contributes to the development of the genetic expression of hypertension.

The purpose of the present study was examine the effects of peripheral sympathectomy on various lymphocyte populations and on the development of hypertension in the SHR. When given to newborn rats, guanethidine sulfate will produce a peripheral sympathectomy while having no effect on central noradrenergic neurons (Johnson et al., 1975). Administration of guanethedine produces a more complete peripheral sympathectomy than neonatal administration of 6-hydroxydopamine and does so with no significant effect on central noradrenergic neurons (Johnson et al., 1975)

Purpose

The purpose of this experiment was to block the development of the sympathetic nervous system in SHR and see if this would reverse any of the alterations in lymphocyte populations previously noted in this strain.

Rationale

The sympathetic nervous system has been shown to exercise control over the immune system. Spontaneously hypertensive rats have an overactive sympathetic nervous system therefore blockade of the system will show if it plays a direct role in the development of lymphocyte changes and blood pressure simultaneously.

Methods

Animals

For guanethidine treatment, male rat pups were obtained on-site from breeding pairs purchased from Charles River Labs, Inc (Wilmington,

Mass). Untreated male SHR and WKY were obtained from Charles River Labs, Inc. at 28 days of age and placed in cages with ad-lib rat chow and water in a room with a 12:12 light:dark cycle.

Guanethidine treatment

Newborn male SHR were given guanethidine sulfate (Sigma Chemical, St. Louis, MO) at a dose of 100mg/kg/day for the first 25 days of life. The injections were made subcutaneously in a vehicle of 0.9% NaCl solution (10ul/gbwt). The guanethidine solution was made fresh daily and adjusted to a pH of 7.0-7.4. After weaning these animals were placed in cages with ad-lib rat chow and water in a room with a 12:12 light:dark cycle.

Blood Samples:

Blood was obtained at approximately one, three and four months of age from each group of animals as described in Chapter 2.

Lymphocyte Preparation

Lymphocytes were separated and prepared for FACS analysis as described in Chapter 2.

White blood cell (WBC) and lymphocyte counts

Total WBC's were counted directly from whole blood using a Coulter Counter (S plus IV, Coulter Electronics, Hialeah, FL). The blood is diluted with an isotonic saline solution. Red blood cells are lysed with a potassium ferrocyanide solution. The remaining white blood cells are counted via a voltage resistance method.

Whole blood smears were stained with Wright-Geimsa stain for lymphocyte determinations. A drop of blood is placed on a coverslip to produce a blood film. The coverslips are then warm air dried. They are

then placed in an ethanol bath for five minutes followed by placement in Wright stain for ten minutes. All coverslips are then rinsed with distilled water. The slips are then placed in Geimsa stain for 15 minutes, rinsed in distilled water, air dried and mounted. The cells are manually counted.

Blood pressure

Direct arterial pressure measurements were made at five months of age in all animals. Under metofane anesthesia, a vinyl catheter was ligated in the left carotid artery. All measurements were recorded after a ten minute stabilization period.

Statistics:

An analysis of variance followed by a Newman-Kuels test was used to compare the means of all groups.

Results

Lymphocyte populations

At one month of age, the guanethidine treated SHR had a significantly higher percentage of T-nonhelper cells than the control untreated SHR (21.1±1.0 vs 17.6±0.4) ($p<0.05$) (Figure 5-1). There was no significant difference between the percentage of T-nonhelper cells found in the guanethidine treated SHR and untreated WKY. Guanethidine treated SHR showed a significantly higher T-helper percentage (57.8±0.8 vs 53.4±1.6) ($p<0.05$) and a lower B-cell percentage (20.3±1.2 vs 28.1±1.7) ($p<0.05$) when compared to WKY. Untreated SHR had a lower percentage of T-nonhelper cells than untreated WKY (17.6±0.4 vs 22.1±1.1) ($p<0.05$).

The results at three months of age are shown in Figure 5-2. The guanethidine treated SHR have a higher percentage of total T-cells than untreated SHR (81.2±2.5 vs 73.4±1.6)) ($p<0.05$). They also possess a higher percentage of T-helper cells (63.2±1.4 vs 52.7±2.1) ($p<0.05$). The three

month old, guanethidine treated SHR had a lower percentage of B-cells than either the untreated SHR or WKY controls (17.0±1.3 vs 21.7±1.3 and 26.5±2.3, respectively) ($p<0.05$). As was the case at one month untreated SHR had a decreased nonhelper percentage relative to WKY (20.4±0.6 vs 28.2±2.8) ($p<0.05$).

At four months of age (Figure 5-3), untreated SHR had a lower T-nonhelper percentage than either WKY or guanethidine treated SHR (14.4±2.0 vs 23.3±0.6 or 26.1±0.5, respectively) ($p<0.05$). The guanethidine treated SHR also had a significantly higher T-nonhelper percentage than the WKY (26.1±0.5 vs 23.3±0.6) ($p<0.05$).

White blood cell and lymphocyte counts

At one month of age there was no significant change in the number of WBCs between any of the three groups examined (Table 5-1). At four months of age, WKY had fewer actual numbers of WBCs and lymphocytes than either untreated SHR or guanethidine treated SHR.

Blood pressure

All blood pressures were recorded vial direct arterial measurements at five months of age (Table 5-1). The untreated SHR had significantly higher pressures than either WKY or guanethidine treated SHR (185.5±16.4 vs 125.1±5.8 and 110±3.0, respectively) ($p<0.05$).

Discussion

The results show that sympathectomy at birth by daily treatment with guanethidine produces SHR rats that do not have high blood pressure as adults. The results also show that when blood pressure is normalized in SHR by this method the T-nonhelper cells of the immune system are not

depressed as they are in untreated SHR and follow the same trend as the T-nonhelper cell numbers in normotensive controls.

The SHR has been shown to possess various immune system alterations that are related to the development of hypertension (Takeichi et al., 1981; Norman et al., 1985; Bendich et al., 1981; Norman and Dzielak, 1986; Khraibi et al., 1984). Another well known characteristic of the SHR is its increased sympathetic nervous system activity (Norman and Dzielak, 1986; Judy et al., 1976). This increase in SNS activity along with an increase in plasma arginine vasopressin (AVP) are also associated with the development of hypertension in this model (Norman and Dzielak, 1986; Judy et al., 1976; Crofton et al., 1978).

The SNS has been shown to influence the immune system (Irwin et al., 1988; Braun et al., 1985). An increase in SNS activity is associated with a decrease in immunocompetence or immune system activity. Our objective was to block peripheral sympathetic activity in the SHR and then determine if any changes in blood pressure or lymphocyte populations occurred.

At both one month and four months of age the level of T-nonhelper cells was significantly greater in the guanethidine treated animals relative to the control untreated SHR. At four months of age the guanethidine treated SHR level was also significantly higher than the WKY. This T-nonhelper cell level appears to be important. SHR have a generalized depression of T-lymphocytes, especially T-nonhelper lymphocytes (Takeichi et al., 1981; Norman et al., 1985; Fernandes et al., 1986). In our lab, this population is depressed as early as two weeks of age, well before the development of hypertension (Chapter 4). The antibody that we used

here for the T-nonhelper cells labels T-suppressor cells, cytotoxic T-cells as well as some natural killer cells. The percentages of the different lymphocyte populations were not reflected in the absolute numbers. Adult WKY animals had significantly less WBC's and lymphocytes than either the untreated control SHR or the guanethidine treated SHR. This is an important observation. It appears to be the relative proportion of one cell type to another that is important and not the absolute number of cells.

In summary, by blocking peripheral sympathetic output in the SHR we have demonstrated a decrease in blood pressure and an alteration in the different lymphocyte populations examined. What appears to be important here is not the absolute number of cells, but the relative proportions of one cell type to another. In a normal situation a balance between the different cell types and their chemical mediators is achieved. When this balance is disturbed, such as by a decrease in a regulatory cell type found in the nonhelper population, an imbalance may occur not only in other cell types but of the chemical mediators they produce. These chemical mediators include substances that are similar, if not identical to ACTH, thyroid stimulating hormone (TSH) and leutenizing hormone (LH) (Naito et al., 1989; Morley et al., 1987; Blalock, 1984). These chemical mediators may then be acting not only locally in an immunomodulatory role, but peripherally and even centrally, to influence the body's homeostatic mechanism.

Guanethidine treatment produces a peripheral sympathetic denervation (Johnson et al., 1975). The rats treated from birth for 25 days did not develop hypertension, confirming the important role of the SNS in the expression of genetic hypertension (Norman and Dzielak, 1986; Judy et al., 1976). Since the lack of sympathetic activation (or of its overactivity)

was associated with the absence of T-nonhelper cell suppression, we can make two conclusions: a) Changes in the immune system in these SHRs is mediated by the SNS which implies a central control over the immune system, and b) T-nonhelper cell suppression and hypertension are related to the presence of an active SNS. The results are relevant to the concept that hypertension is expressed when the immune system is suppressed and the SNS is overactive.

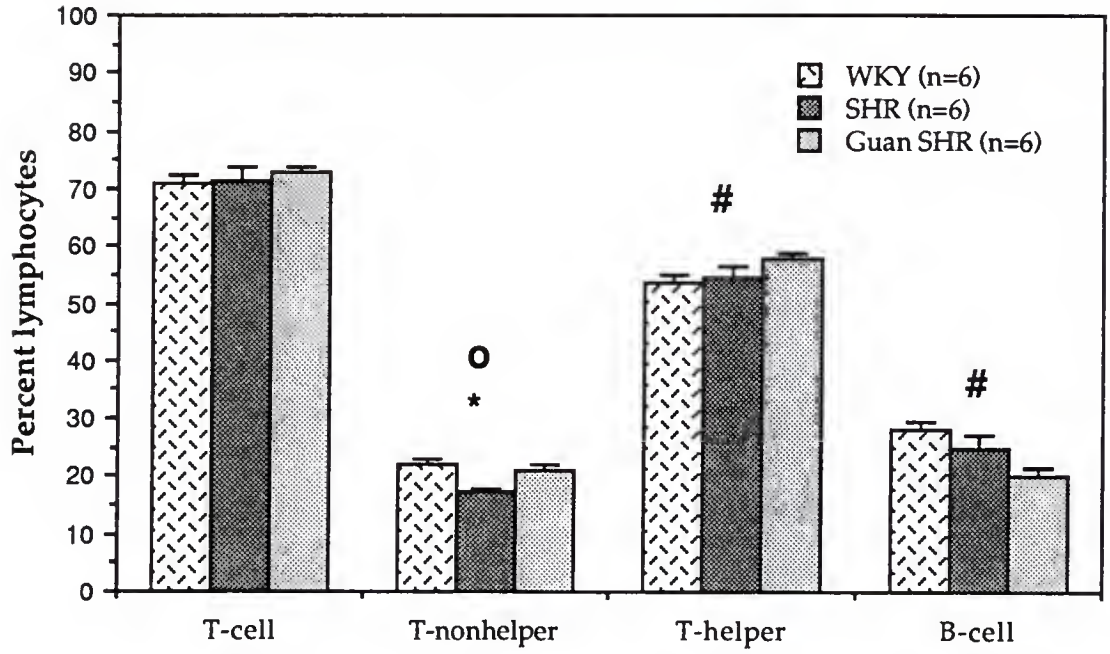

Figure 5-1: Differences between lymphocyte populations at one month of age. All values are expressed as Mean ± SEM. * = $p<0.05$ for Guan SHR vs SHR. # = $p<0.05$ for Guan SHR vs WKY. o = $p<0.05$ for SHR vs WKY.

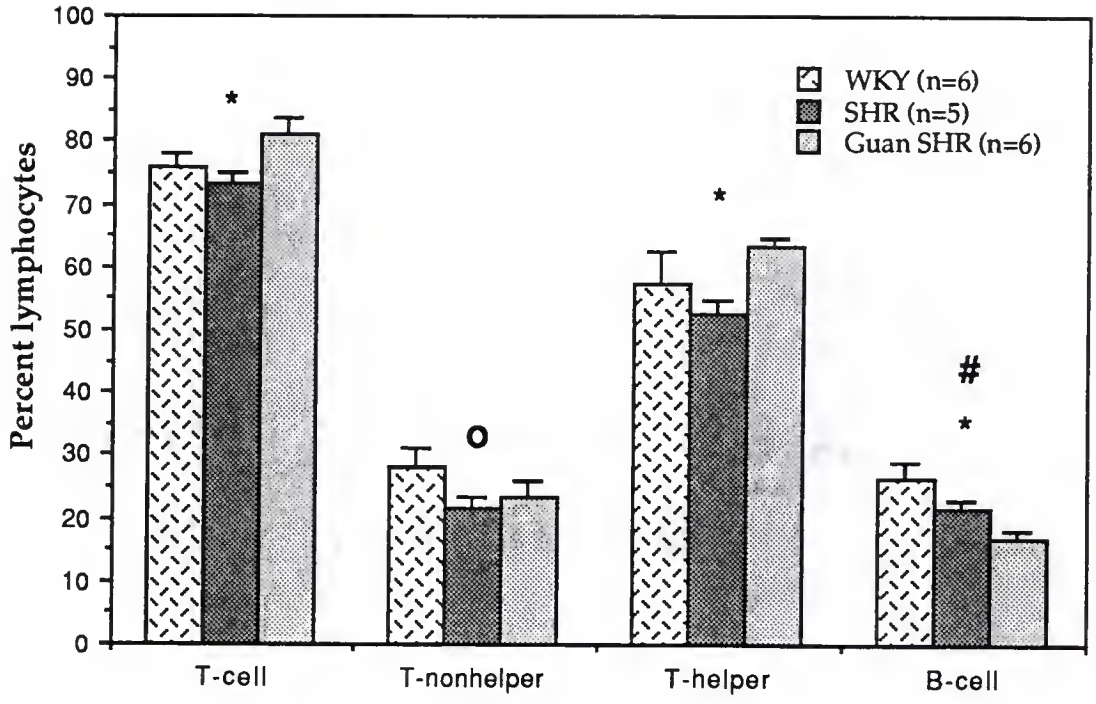

Figure 5-2: Differences between lymphocyte populations at three months of age. All values are expressed as mean ± SEM. * = $p<0.05$ for Guan SHR vs SHR. # = $p<0.05$ for Guan SHR vs WKY. o = $p<0.05$ for SHR vs WKY.

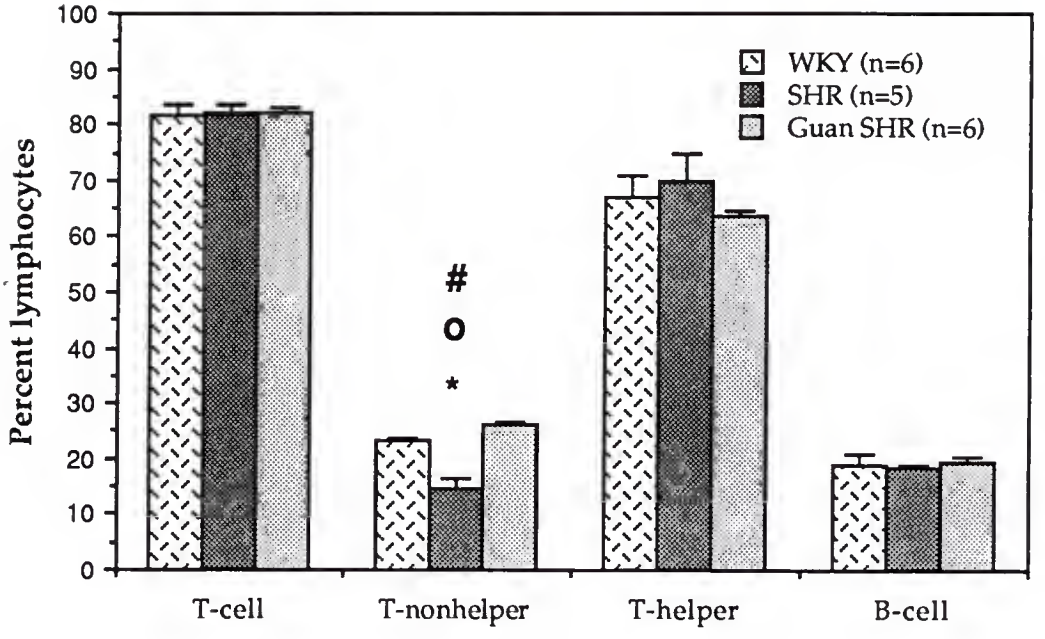

Figure 5-3: Differences between lymphocyte populations at four months of age. All values are expressed as mean ± SEM. * = $p<0.05$ for Guan SHR vs SHR. # = $p<0.05$ for Guan SHR vs WKY. o = $p<0.05$ for SHR vs WKY.

TABLE 5-1:
LEUKOCYTE (per ul blood) AND BLOOD PRESSURE (mmHg) DATA FOR GUANETHIDINE TREATED SHR, UNTREATED CONTROL SHR AND UNTREATED WKY

	N	1 month WBC	4 month WBC	4 month lymphocytes	5 month BP
WKY	6	4550±679	6767±210*+	5032±219*+	125.1±5.8
SHR	5	6570±1040	10140±609	7982±550	185.5±16.4
Guan SHR	6	5970±308	10183±588	7038±241	110±3.0

All values expressed as Mean ± SEM.

* = $p<0.05$ for SHR vs WKY
+ = $p<0.05$ for guan SHR vs WKY

All blood pressures on this table are direct arterial measurements.

CHAPTER 6
INTERLEUKIN-2 TREATMENT ALTERS LYMPHOCYTE POPULATIONS, BUT NOT BLOOD PRESSURE, IN SPONTANEOUSLY HYPERTENSIVE RATS

Introduction

The spontaneously hypertensive rat (SHR) has been given much attention as a model of human essential hypertension. The exact cause or causes of the hypertension in this model remain to be fully understood. Among the factors considered likely are an increase in central Angiotensin II (Ang II) (Phillips and Kimura, 1988), an increased sympathetic nervous system (SNS) activity (Norman and Dzielak, 1986; Judy et al., 1976; Norman and Dzielak, 1982) and an increase in arginine vasopressin (AVP) secretion (Crofton et al., 1978). Another factor that has been gaining more attention in this model is the immune system (Norman and Dzielak, 1986; Bendich et al., 1981; Takeichi et al., 1986; Norman et al., 1985; Takeichi et al., 1981). The SHR has been shown to have fewer and less active T-cells (Takeichi et al., 1981; Takeichi et al., 1986). Thymic implants from WKY into neonatal SHR can attenuate the hypertensive response (Norman and Dzielak, 1986; Takeichi et al., 1986; Norman et al., 1985). Also, chronic immunosuppression with cyclophosphamide attenuates hypertension in the SHR (Khraibi et al., 1984). Work in our own lab has shown that SHR exhibit a decrease in the percentage of T-nonhelper cells both in the prehypertensive and hypertensive phases of development (Chapter 4).

Recently, Tuttle and Boppana (1990) showed that a single bolus injection of IL-2 given to young SHR could completely normalize blood pressure for at least two months. IL-2 is a lymphokine that is involved in the differentiation and proliferation of T-cell subpopulations. Work in our lab has focused on the balance of the different lymphocyte populations and how an alteration of this balance may set the stage for the initiation of various disease processes. Given the fact that we have found alterations in the percentages of different lymphocyte populations in SHR (Chapter 4) we decided to repeat the Tuttle and Boppana study and again examine these same parameters.

Purpose

The purpose of this experiment was to examine the effect of interleukin-2 on the development of hypertension in SHR. Another goal was to test the effect of the treatment on the lymphoycte populations that we have previously examined in this strain.

Rationale

The immune system has been implicated in the pathogenesis of hypertension in SHR. A previous study showed that treatment with IL-2 could abolish the development of high blood pressure in this model. We are therefore testing the finding and also testing if the immune response and blood pressure response to IL-2 are correlated.

Methods

Animals

Twenty-eight day old SHR and WKY were obtained from Charles River Labs, Inc. (Wilmington, MA). They were housed in wire bottom cages in a

temperature controlled room with a 12 hour light, 12 hour dark cycle. All animals were given access to ad-lib rat chow and water.

The rats were divided into four groups. SHR were divided into a control (n=5) or treatment group (n=8, IL-2 treatment). WKY were also divided into control (n=6) or treatment (n=8, IL-2 treatment) groups.

IL-2 treatment:

Human recombinant IL-2 was purchased from Cell Products, Inc. (Buffalo, NY) and a working solution was prepared by dilution with Macrodex 6% in normal saline (Pharmacia Labs, Piscataway, NJ). At 42 days of age all animals that were to recieve IL-2 were individually weighed and given a single, subcutaneous bolus injection of 5000 units/kg of IL-2.

Blood preparation

Blood was obtained at approximately three and four months of age from each group of animals as previously described in Chapter 2.

Lymphocyte Preparation:

Lymphocytes were separated and prepared as described in Chapter 2.

Blood pressure

Systolic blood pressures were obtained in unanesthetized, restrained animals using a tail plethysmograph. Readings were obtained from all animals at two months of age and again at four and a half months of age.

White blood cell (WBC) and lymphocyte counts:

Total WBC's were counted directly from whole blood using a Coulter Counter (S plus IV, Coulter Electronics, Hialeah, FL) and whole blood smears were stained with Wright-Geimsa for lymphocyte determinations as described in Chapter 5.

Statistics

The means of all groups were compared using an analysis of variance followed by a Newman-Kuels test.

Results

Blood pressures and body weight

At two months and four and a half months control SHR and IL-2 treated SHR had significantly increased blood pressure relative to control WKY and IL-2 treated WKY, respectively (Table 6-1). At neither age were the blood pressures for the control SHR and IL-2 treated SHR significantly different.

All rats were weighed at four and a half months. IL-2 treated WKY weighed more than IL-2 treated SHR ($p<0.05$). There was no significant weight difference between the control SHR and WKY or between control SHR and IL-2 treated SHR.

Lymphocyte populations:

All values are expressed as a percentage of total lymphocytes examined. At three months of age (Table 6-2) both the control SHR and IL-2 treated SHR showed a decrease in the T-nonhelper cell population compared to the control WKY and IL-2 treated WKY, respectively ($p<0.05$). IL-2 treated SHR also showed a decrease in the percentage of B-cells present when compared to IL-2 WKY ($p<0.05$). The IL-2 treated SHR had a significant decrease in the percentage of B-cells when compared to control SHR ($p<0.05$).

At four month of age (Table 6-3) the picture changes somewhat. The control SHR animals show a decreased percentage of T-nonhelper cells relative to the WKY controls ($p<0.05$). The IL-2 treated SHR also continue to show a decrease in the T-nonhelper population percentage in relation

to the IL-2 treated WKY ($p<0.05$). The IL-2 treated SHR showed an increase in the percentage of T-nonhelper cells and a decrease in the percentage of T-helper cells in relation to the control SHR ($p<0.05$). IL-2 treated SHR retained the decrease in the B-cell percentage compared to the control SHR that was seen at 3 months of age. IL-2 treated WKY also had a decrease in the T-helper population relative to control WKY ($p<0.05$).

White blood cell and lymphocyte counts

At four months of age the control SHR and IL-2 treated SHR had significantly greater numbers of WBCs and lymphocytes than the control WKY and IL-2 treated WKY (Table 6-4). There was no significant difference between the IL-2 treated animals and the control animals for either SHR or WKY.

Discussion

Tuttle and Boppana (1990) recently reported that a single bolus injection of IL-2 in young SHR could abolish the development of hypertension. The results that we present here do not support this finding. We found no difference in the blood pressure of SHR treated with a single dose of IL-2 compared to untreated SHR at two months or four and a half months of age. IL-2 does have effects on the immune system of the SHR.

IL-2 is a lymphokine that has important functions in the immune response. It is secreted by T-lymphocytes, especially stimulated T-helper lymphocytes. Once secreted it can act on other T-lymphocytes as well as B-lymphocytes to increase proliferation and differentiation. Evidence is emerging to indicate that IL-2 has other non-immune system related functions. Peripherally, IL-2 has been shown to have a positive inotropic effect on isolated rat atria (Elizalde de Bracco et al., 1989). Central nervous

system effects have been observed as well. When injected intracerebroventricularly, IL-2 causes behavioral sedation in rats. This effect is blocked by naloxone, indicating that it is mediated via opiate receptors (De Sarro et al., 1990). There is evidence that IL-2 can increase the number of neonatal rat sympathetic neurons in culture and that sympathetic neurons may have IL-2 receptors (Haugen and Letourneau, 1990). IL-2 binding sites have been found in the rat hippocampus, and these binding sites may be important modulators of cholinergic activity and release (Araujo et al., 1989a). IL-2 like material has also been isolated from the rat hippocampus, striatum and frontal cortex (Araujo et al., 1989b). Significantly, IL-2 has been shown to cause ACTH release from pituitary cells and to raise plasma ACTH (Smith et al., 1989; Naito et al., 1989). Studies in humans have also pointed to a central role for IL-2. Cancer patients given IL-2 show behavioral and neurologic changes (Denicoff et al., 1987; Kolitz et al., 1988; Kakumu et al., 1988). Patients with progressive multiple sclerosis and chronic fatigue syndrome show similar neuropsychiatric effects as those cancer patients on IL-2 therapy. Current studies have shown that these patients have higher than normal values of serum IL-2 (Trotter et al., 1988; Cheney et al., 1989). Even though the previously mentioned data suggests an important role for IL-2, its exact function in maintaining normal body homeostasis is unknown.

Studies from our lab have shown that the SHR exhibit decreases in the percentage of T-nonhelper cells from a very early age through the development and establishment of hypertension (Chapter 4). Our present study confirms with this observation. Both the three and four month old control SHR and IL-2 treated SHR showed decreases in the percentage of

T-nonhelper cells relative to WKY and IL-2 treated WKY, respectively (Tables 6-2 and 6-3). At four months of age the IL-2 treated SHR also showed a significant decrease in the percentage of B-cells (Table 6-3) also in agreement with our previous study (Chapter 4).

Our IL-2 treated SHR showed no significant decrease in blood pressure relative to untreated SHR. This is in direct conflict with the results of Tuttle and Boppana (1990). We used the same age of rats, dose of IL-2 and measured BP for the same time period. Hypertension developed equally in the two SHR groups. Our rats were obtained from a different source that those used in the Tuttle and Boppana (1990) study. This could account for some of the observed differences including the failure of our SHR to have an attenuated blood pressure response following IL-2 administration. The IL-2 was active because we observed differences in the lymphocyte populations we examined between the IL-2 treated SHR and the untreated, control SHR. At three months of age, the IL-2 treated SHR showed a significant decrease in the number of B-cells. At 4 months of age this difference was maintained along with a decrease in the percentage of T-helper cells and an increase in the number of T-nonhelper cells. It is possible, however, that the IL-2 used in these experiments was less active than that used by Tuttle and Boppana (1990)

The T-nonhelper population appears to be important for the expression of hypertension. As mentioned previously, studies in our lab show this cell population is depressed as early as two weeks of age in SHR (Chapter 4). This finding may be generalizable as this cell population is also decreased in inbred prehypertensive Dahl salt-sensitive rats (Chapter 8). The nonhelper antibody that we have used here labels cytotoxic T-lymphocytes, suppressor T-lymphocytes as well as some natural killer

cells. A reduction in the activity of T-suppressor cells is often associated with autoimmune disease (Eisen, 1980). SHR have been shown to have reduced T-lymphocyte function as well as reduced thymus weight (Takeichi et al., 1986; Takeichi et al., 1981). The present findings and those of others point to an altered immune system being at least a partial cause of the development of hypertension in the SHR (Bendich et al., 1981; Takeichi et al., 1986; Norman et al., 1985; Takeichi et al., 1981; Khraibi et al., 1984).

Our study does not offer any evidence as to the cause of the observed alteration in the different lymphocyte populations. However, IL-2 actions on CNS development and output and IL-2 action on adrenal steroid production and secretion are two possibilities.

The data presented show that both groups of SHR exhibited increased numbers of lymphocytes and WBCs. Previous studies in this area are somewhat confusing. One study showed SHR as having a decrease in the amount of WBCs (Tadeichi et al., 1981). Our data agrees with a later study showing SHR having an increase in the number of WBC's (Takeichi et al., 1986). Both of these previous studies show the amount of lymphocytes to be statistically unchanged in the SHR. It is difficult to speculate on the contradictory results found in our study and the two previously mentioned studies.

This raises an important question. Is it the absolute number of lymphocytes that is important? It is more likely that the major factor involved is the proportion of one cell type to another. In a normal situation the cells of the immune system communicate with each other via their respective cytokines. In this case a balance is achieved. If the

proportions of the different cell types are altered in some way the balance of chemical mediators can be altered. As has been suggested these cytokines can have important non-immunologic roles and may be influencing the body's homeostatic regulating system.

In summary, we present data showing that a bolus IL-2 injection at 42 days of age did not attenuate the development of hypertension in SHR but did affect the dynamics of the different lymphocyte populations observed.

TABLE 6-1:
SYSTOLIC BLOOD PRESSURE (mm Hg) AND BODY WEIGHT (g) FROM IL-2 AND CONTROL SHR AND WKY

	N	2 month BP	4.5 month BP	4.5 month BW
WKY	6	130±6.4+	135±6.0+	350±5.1
IL-2 WKY	8	124±4.5*	127±2.1*	367±5.4*
SHR	6	152±5.3	187±4.8	331±6.7
IL-2 SHR	8	145±4.2	178±3.2	337±8.6

All values expressed as Mean ± SEM
* = $p<0.05$ for IL-2 SHR vs IL-2 WKY
+ = $p<0.05$ for SHR vs WKY

TABLE 6-2:
PERCENTAGE OF DIFFERENT LYMPHOCYTE POPULATIONS AT THREE MONTHS OF AGE

	N	T-cell	T-nonhelper	T-helper	B-cell
WKY	6	75.8±2.2	26.0±1.9&	57.4±5.1	26.5±2.3
IL-2 WKY	8	82.1±3.0	24.1±0.6*	58.0±2.4	23.0±1.3*
SHR	5	73.4±1.6	20.4±0.6	52.7±2.1	21.7±1.3+
IL-2 SHR	8	77.5±1.6	21.6±0.4	56.4±1.8	17.6±0.5

All values expressed as Mean ± SEM
* = $p<0.05$ for IL-2 SHR vs IL-2 WKY
+ = $p<0.05$ for IL-2 SHR vs SHR
& = $p<0.05$ for SHR vs WKY

TABLE 6-3:
PERCENTAGE OF DIFFERENT LYMPHOCYTE POPULATIONS AT FOUR MONTHS OF AGE

	N	T-cell	T-nonhelper	T-helper	B-cell
WKY	6	81.4±2.4	23.3±0.6&	67.2±3.6#	18.7±2.3
IL-2 WKY	8	81.1±1.7	22.1±0.5*	56.9±1.2	19.3±0.8*
SHR	5	82.0±1.5	14.4±2.0+	70.0±4.8+	18.3±0.7+
IL-2 SHR	8	78.7±0.8	19.0±0.8	58.7±0.6	16.2±0.3

All values expressed as Mean ± SEM
* = $p<0.05$ for IL-2 SHR vs IL-2 WKY
+ = $p<0.05$ for IL-2 SHR vs SHR
# = $p<0.05$ for IL-2 WKY vsWKY
& = $p<0.05$ for SHR vs WKY

TABLE 6-4:
WHITE BLOOD CELL AND LYMPHOCYTE COUNTS (per ul blood) AT FOUR MONTHS OF AGE

	N	4 month WBC	4 month lymphocyte
WKY	6	6767±254+	5032±223+
IL-2 WKY	8	6650±246*	4619±143*
SHR	5	10140±609	7982±550
IL-2 SHR	8	9813±260	7520±268

All values expressed as Mean ± SEM
* = $p<0.05$ for IL-2 SHR vs IL-2 WKY
+ = $p<0.05$ for SHR vs WKY

CHAPTER 7
ANGIOTENSIN II LEVELS IN THE SPLEENS OF THREE RAT STRAINS

Introduction

Angiotensin II (Ang II) is an important peptide in the hemodynamic control mechanisms of the body. It is produced in the blood by the enzymatic cleavage of Angiotensin I by converting enzyme. This circulating Ang II is a vasoconstrictor and exerts its actions on vascular beds throughout the body. Besides these direct effects Ang II also influences blood pressure and fluid balance through potentiating the effects of norepinephrine and stimulating aldosterone release from the adrenal cortex (Zimmerman et al., 1987).

Recently, a possible role of Ang II has been suggested in influencing the immune system. Ang II binding sites have been localized in the rat spleen as well as in isolated rat and mouse spleen cells (Castren et al., 1987; Weinstock and Kassab, 1986). Granuloma macrophages in murine schistosomiasis have been shown to produce Ang II. This Ang II is chemotactic for T and B lymphocytes (Weinstock and Kassab, 1986).

The spontaneously hypertensive rat (SHR) is an accepted model of human essential hypertension. Abnormalities in the immune system of this model have been suggested as a possible cause of the hypertensive state. Thymic implants from WKY into neonatal SHR will attenuate the development of hypertension (Norman and Dzielak, 1986; Norman et al., 1985). SHR given immunosuppressive drugs will also have an attenuated

hypertension (Khraibi et al., 1984). It appears that this model has a depression of T-lymphocytes, especially the T-suppressor cell population (Norman et al., 1985; Takeichi et al., 1981). Interestingly, the SHR also shows an increased level of brain Ang II (Phillips and Kimura, 1988).

The goal of the present study was to look at levels of Ang II in the spleen, an important immune organ. The spleens of SHR, WKY and Sprague-Dawley (SD) rat strains were examined. The SHR was chosen because of the large amount of data concerning immunodeficiency in these hypertensive animals. The other strains were used as normotensive controls.

Purpose

The goal of this study was to measure angiotensin II in the spleen of the rat. A secondary goal was to test if the Ang II levels differed from one strain to another.

Rationale

Angiotensin II binding has been found in the spleen but no previous study has measured Ang II in the spleen. The Ang II content of many tissues differs among different rat strains. Therefore, we predicted that Ang II would be present in the spleen.

Methods

Animals

Age matched, male SHR, WKY and Sprague Dawley rats were obtained at four months of age from Charles River Labs, Inc. (Wilmington, MA). All animals were placed in a temperature controlled room with a 12 hour light, 12 hour dark cycle. All animals were placed in wire bottom cages,

with access to ad-lib water and rat chow (#5001, Purina Mills, St. Louis, MO).

Extraction

The rats were anesthetized with Metofane anesthesia (Pitman-Moore) and decapitated. An incision was made over the left abdominal area and the spleen dissected out. The spleen was then placed in saline solution and any excess fat removed. All spleens were then lightly blotted dry, weighed, cut into three pieces and placed in a mixture of dry ice and isopentane for snap freezing. All spleens were stored at -80°C.

Individual frozen spleen chunks were weighed and placed in ten volumes of 1M acetic acid heated to 125°C for 20 minutes, to denature proteins and prevent hydrolysis. All samples were then homogenized using an Ultra-Turrax (Tekmar Co., Cincinnati, OH). The homogenate was then spun at 10,000g for 20 minutes and the supernatant poured off. The remaining pellet was resuspended in one ml of 1M acetic acid and centrifuged as before. This supernatant was added to the first supernatant.

SepPak C-18 Purification

For preparation of tissue for RIA and HPLC, SepPak C-18 cartridges were used. The cartridges were moistened with 3ml of methanol and washed with ten ml of 1% trifluoroacetic aced (TFA) in water, then coated with one ml of 1% Polypep solution (Sigma Chemical, St Louis, MO) in water to protect against nonspecific absorption of angiotensin on the cartridge, and washed with ten ml of methanol, water, and TFA (80/90/1, vol/vol), followed by ten ml of 1% TFA in water.

The spleen supernatant was applied to the SepPak cartridge and the tube was rinsed with ten ml 1% NaCl/1% TFA(vol/vol) which was also applied. The cartridge was washed twice with five ml of 1% TFA/1% NaCl

(1/1, vol/vol), and two ml of methanol/water/TFA (30/69/1, vol/vol). Peptides retained in the cartridge were eluted with methanol/water/TFA (70/29/1, vol/vol). The eluate was dried under air in polypropylene tubes on a warm plate.

High pressure liquid chromatography (HPLC) and radioimmunoassay (RIA)

HPLC elutions were performed on spleen samples from SHR and WKY as well as 3H-AngII for the purpose of confirming the coelution of an Ang II-like peptide in the spleen with synthetic Ang II. This was accomplished using a Beckman HPLC model 332 with a method described previously (Phillips and Stenstrom, 1985).

RIA was performed on all samples for the quantification of Ang II. This was accomplished using the method of Phillips and Stenstrom (1985).

Statistics

Where two means were being compared Student's t test was used. When the means from 3 groups were being compared an analysis of variance followed by a Newman-Kuels test was performed.

Results

Spleen weights

As is shown in Table 7-1, Spleens from the SHR animals weighed significantly more than the WKY animals (0.610±0.047g vs 0.514±0.030g).

HPLC elution profile

Figure 7-1 shows that the immunoreactive Ang II found in SHR and WKY spleens coelutes in the same fraction as that for 3H-Ang II.

Standard curve for RIA

To make sure that there was no non-specific interference from the spleen tissue, a SepPak purified WKY spleen extract was serially diluted and compared to the Ang II standard curve (Figure 7-2). The slope of the sample is parallel to that of the standard curve indicating no non-specific interference.

Levels of Ang II in different rat strains

Figure 7-3 shows the results of quantifying the Ang II levels from the spleens of SHR, WKY and Sprague-Dawley rats. Interestingly, the WKY had significantly less Ang II/g tissue than either the SHR or Sprague-Dawley (505.2±24 pg Ang II/g tissue versus 734.9±78 and 854.0±68, respectively) ($p<0.05$).

Discussion

The results presented here demonstrate that there are high levels of Ang II in the rat spleen. The Ang II that was extracted from the rat spleen elutes in the same fraction as that for synthetic Ang II. Control tubes that contained no tissue showed no evidence of Ang II.

Not only was Ang II found in the spleen, but the levels, in the hundreds of picograms could not be accounted for merely by the presence of blood in the tissue. Typical plasma levels of Ang II are in the range of 100pg/ml (Van Eekelen and Phillips, 1988). Clearly, these high levels are not just a reflection of plasma Ang II flowing through the spleen tissue.

The spleen is an important immune organ. It has dense populations of macrophages as well as T and B lymphocytes. Although the white blood cell (WBC) arrangement in the spleen is a very dynamic thing, the different cell types (macrophages, T-lymphocytes and B-lymphocytes) do

tend to be found in generally specific locations within the splenic structure.

It has been shown that macrophages can produce Ang II. This Ang II is chemotactic for both T and B lymphocytes (Weinstock and Kassab, 1986). It is further suggested that this chemotactic activity is mediated via high affinity Ang II receptors (Weinstock and Kassab, 1986). This fits in with studies done by other investigators using autoradigraphy who have shown the presence of Ang II binding sites in rat spleen sections as well as in isolated rat spleen cells (Castren et al., 1987).

The SHR is often used as a model of human essential hypertension. Several investigators have linked abnormalities of the immune system to the development of this hypertensive state. SHR given the immunosuppressive drug, cyclophosphamide, show an attenuated development of hypertension (Khraibi et al., 1984). Neonatal SHR implanted with WKY thymic tissue also show an attenuated hypertensive response (Norman and Dzielak, 1986; Norman et al., 1985). Finally, SHR show an immunological depression, suggestive of impaired T-lymphocyte activity (Takeichi et al., 1981).

The SHR also shows an increased sympathetic nervous system activity (Judy et al., 1976). The norepinephrine content per gram of spleen tissue is higher in young SHR, possibly due to an increased sympathetic innervation (Donohue et al., 1988). This also might partially explain the decrease in blood flow rate to the spleen of SHR (Kimura et al., 1988).

Our study found an increased Ang II content in the spleens of SHR versus WKY. This fact may help explain the above observations. Ang II potentiates the release and action of norepinephrine from sympathetic nerve terminals (Zimmerman et al., 1987). The WKY spleen Ang II

content was also decreased relative to the other normotensive control, the Sprague-Dawley rat. It is difficult to speculate on the reason behind this finding, although variations among strains are sure to play at least some role. This also brings up the possibility that these relatively low levels of Ang II in the spleens of WKY are just a unique characteristic of this strain. This would imply that the levels found in SHR and Sprague-Dawley rats are "normal".

We have previously shown that the SHR exhibits a decrease in the percentage of T-nonhelper lymphocytes from as early as two weeks of age (Chapter 4). Other investigators, as well, have noted a T-cell depression, especially of T-suppressor lymphocytes in the SHR (Norman et al., 1985; Takeichi et al., 1981). Increases in SNS activity have been linked to decreased in immune activity (Irwin et al., 1988; Braun et al., 1985). Even with these findings we have also found an increase in total WBCs and lymphocytes in SHR relative to WKY (Chapter 5). This may partially explain the increased spleen weight we noted in SHR. It is possible to hypothesize that increased proliferation of some immunocompetent cell types, brought about by a reduction in the T-suppressor cell population, for example, may be causing this increase in spleen weight.

In summary, we have shown significant quantities of Ang II in the rat spleen. Furthermore, these amounts were significantly higher in SHR and SD rats than WKY animals. Whether this increase in SHR is due to immune system dysfunction in these animals remains to be demonstrated. However, it is reasonable to assume that this splenic Ang II is related to immune system function and that this interaction may be in some part responsible for the development of the hypertensive state.

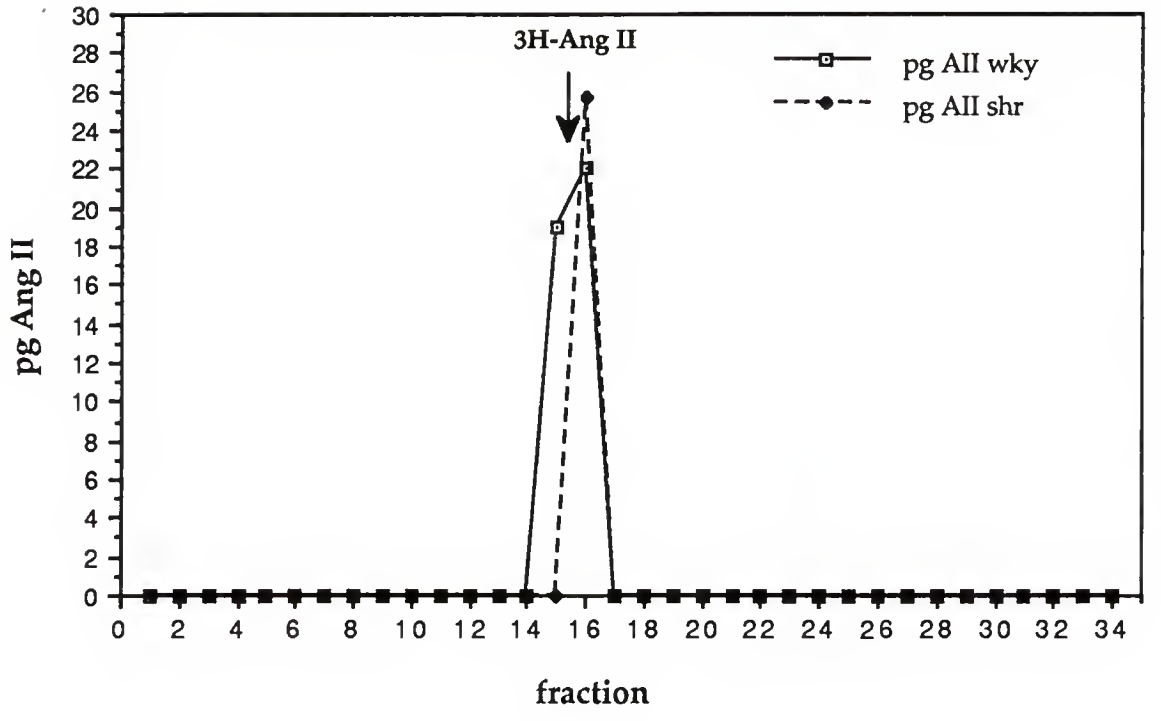

Figure 7-1: HPLC elution profile of an Ang II like peptide in the spleens of a SHR and WKY rat. The fraction at which 3H-Ang II eluted is also indicated. This figure demonstrates tat the Ang II-like material coeluted with synthetic Ang II.

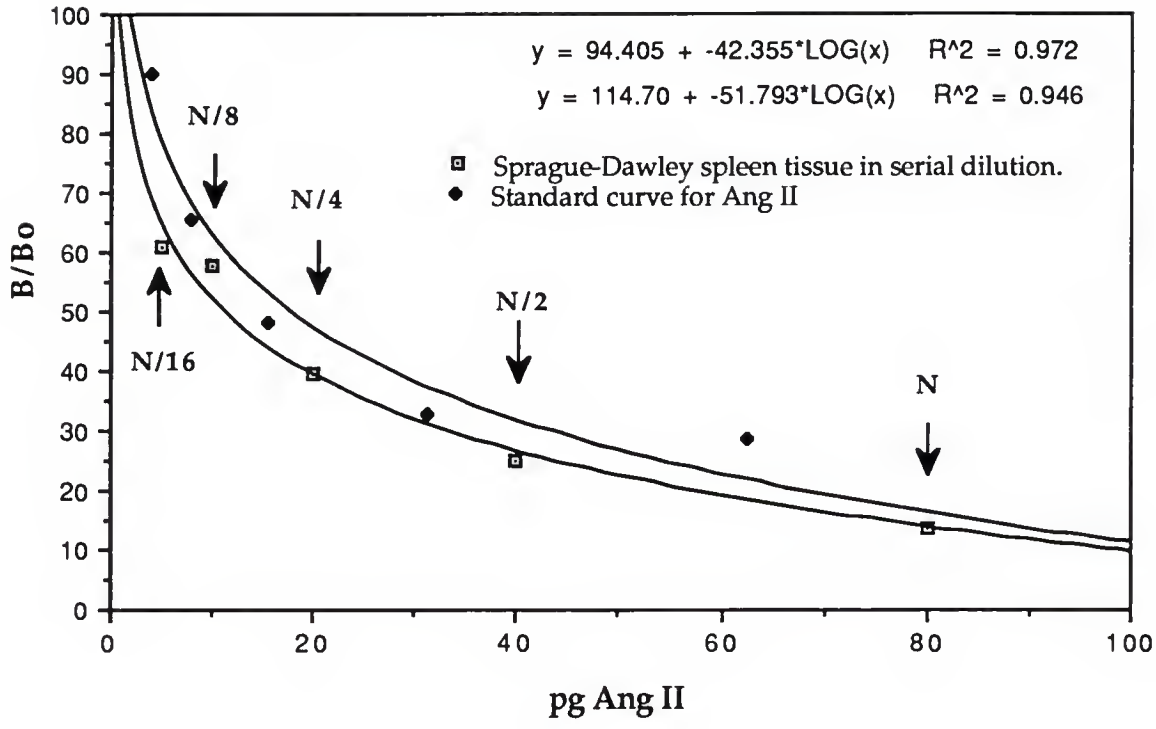

Figure 7-2: Standard curve for Ang II RIA. To make sure there was no nonspecific interference from the spleen tissue a purified extract was serially diluted and the slope of the sample was compared to that of the standard curve. Serial dilution of the sample gives a slope parallel to that of the sample, indicating no interference. N = 100ul of purified spleen extract.

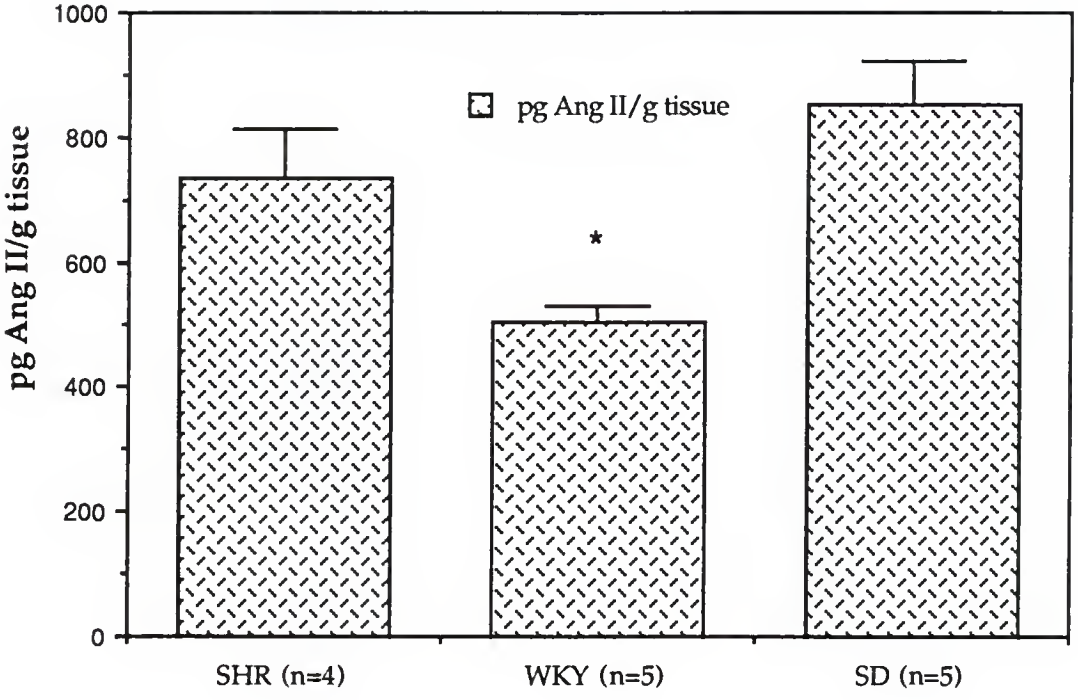

Figure 7-3: Amount of Ang II per gram spleen tissue from SHR, WKY and Sprague-Dawley (SD) rats. * = $p<0.05$ for SHR and SD versus WKY. Values are Mean±SEM.

TABLE 7-1:
COMPARATIVE SPLEEN WEIGHTS FOR SHR and WKY ANIMALS

	N	Spleen Weight (g)
SHR	7	0.610±0.02
WKY	7	0.514±0.01*

All values are Mean ± SEM. * = $p<0.05$ for SHR vs WKY.

CHAPTER 8
ALTERATIONS IN LYMPHOCYTE POPULATIONS OCCUR PRIOR TO THE DEVELOPMENT OF HYPERTENSION IN INBRED DAHL RATS

Introduction

The Dahl strain of rat is a commonly used model of hypertension. When fed a high sodium diet Dahl salt sensitive (DS) rats become hypertensive, but Dahl salt resistant (DR) rats do not. The original strain developed by L.K. Dahl was not an inbred strain and this has produced a certain amount of confusion concerning the role of this animal as an adequate model of hypertension. This problem was overcome by the development of an inbred strain of Dahl S and R rats from the original lines by Dr. J. P. Rapp (Rapp and Dene, 1985). These strains are referred to as S/JR and R/JR for those rats that become rapidly hypertensive when exposed to a high sodium diet and those that do not, respectively. Both the inbred and outbred strains share similar characteristics.

Although many characteristics have been looked at in these animals, little attention has been focused on their immune systems. The immune system has been implicated in the pathogenesis of hypertension in humans (Svendson, 1979; Raff and Wortis, 1970; Mathews et al., 1974). There is also some provocative data showing that immune abnormalities are at least in part responsible for the development of hypertension in the spontaneously hypertensive rat (SHR), another established model of hypertension (Norman et al., 1985; Norman and Dzielak, 1986; Khraibi et al., 1984). Data from our lab indicate that the SHR demonstrates

alterations in the percentage of different lymphocyte populations in both the prehypertensive and hypertensive phases of development (Chapter 4). Specifically, SHR have a decrease in the percentage of T-nonhelper cells from the age of two weeks through at least 4 months.

Lymphocytes are an important effector cell of the immune system. They are capable of secreting chemical mediators, lymphokines. These lymphokines include many different groups of substances such as neuropeptide-like substances (Blalock, 1984; Smith and Blalock, 1985), local acting substances (Grant et al., 1979) and other immunoregulatory substances. It is through these lymphokines and other chemical mediators that the various cells of the immune system communicate with each other. The role of these chemicals in regulating normal body homeostasis remains speculative at best.

Because very small amounts of lymphokines may have very large effects on the proliferation and activity of other lymphocyte sub-populations, it is possible that very subtle changes in these different cell populations can have large actions throughout the entire system. Given this information we decided to look at different lymphocyte populations throughout the development of hypertension in S/JR rats compared to R/JR rats, both before and after the administration of a high (8%) sodium diet

Purpose

The purpose of this experiment was to analyze lymphocyte populations in the prehypertensive as well as hypertensive phases of development in Dahl S/JR rats.

Rational

Immune system abnormalities were found in the development of hypertension in the SHR. The Dahl S/JR rat shares some common characteristics with the SHR such as an increased SHR activity and an increase in AVP secretion. Therefore, the Dahl rat was chosen to test if our finding in SHR was unique or generalizable to another genetic hypertensive model.

Methods

Animals

Male, R/JR (n=6) and S/JR (n=8) were obtained at 28 days of age from Harlan Sprague-Dawley. The animals were placed in wire bottom cages in a temperature controlled room with a 12 hour light, 12 hour dark cycle. Upon arrival all animals received ad-lib water and standard rat chow (#5001, Purina Mills, St. Louis, MO).

Following the initial blood sampling period at 33 days of age all rats were put on an ad-lib high (8%) sodium diet (Purina Mills, St. Louis, MO).

Blood preparation

Blood was obtained at approximately 1 month of age (prior to high sodium diet) and at monthly intervals thereafter. The same group of R/JR and S/JR animals was used throughout the remainder of the study. Under metofane anesthesia the left femoral vein was exposed and a 25 gauge butterfly scalp vein infusion set was used to pierce the vein and obtain one milliliter of blood as described in Chapter 2

Lymphocyte Preparation:

Lymphocytes were separated and prepared as described in Chapter 2.

Blood pressure determination

Systolic blood pressure measurements were made at three months of age in unanesthetized, restrained animals using a tail plethysmograph.

Statistics

The means of both groups were compared using Student's t test.

Results

Lymphocyte populations

At one month of age prior to placing the animals on a high salt diet the S/JR group showed a significant decrease in the percentage of all three T-lymphocyte populations examined (Total T-cells, T-nonhelper and T-helper) ($p<0.05$). A significant increase was also noted in the percentage of B-cells ($p<0.05$) (Figure 8-1).

At two months of age, the animals had been on a high (8%) sodium diet for one month. We once again saw a reduction in the percentage of all T-cell populations ($p<0.05$) in the S/JR group. The percentage of B-cells was still elevated in the S/JR animals relative to the R/JR group ($p<0.05$) (Figure 8-2).

By three months of age only four S/JR animals remained. All rats had been on a high (8%) sodium diet for two months. We again saw that the S/JR animals had a decrease in the percentage of T-cells and T-nonhelper cells ($p<0.05$). At this time point no significant difference was noted in the T-helper populations. The S/JR retained the higher percentage of B-cells at this point ($p<0.05$) (Figure 8-3). The high mortality of S/JR rats that we observed is not uncommon following placement on the high sodium diet (Rapp and Dene, 1985).

Blood pressure

Blood pressure measurements were made at three months of age. Systolic BP were higher in the S/JR animals than the R/JR animals (202±8.6 vs 135±7.5) (Table 8-1).

Discussion

The results of our experiment show that the S/JR rats showed alterations in all lymphocyte populations in the prehypertensive as well as hypertensive phases of development. Importantly these changes were noted prior to placing the animals on a high sodium diet. It is a common misconception that Dahl salt sensitive type rats will develope hypertension only when placed on a high salt diet. This is not the case. The original outbred variety of DS rats as well as the inbred S/JR strain will develope high blood pressure on a low salt diet. The hypertension occurs much more rapidly on a high salt diet (Rapp and Dene, 1985).

The T-nonhelper population percentage was lowered throughout the entire experimental period (Figures 8-1 to 8-3). This is in agreement with our results in the SHR. The SHR exhibit a decrease in the percentage of T-nonhelper cells from as early as two weeks of age (Chapter 4). Alterations in the immune system of the SHR have been implicated in the pathogenesis of their hypertension (Norman et al., 1985; Norman and Dzielak, 1986; Khraibi et al., 1984; Takeichi et al., 1981; Bendich et al., 1981). Thymic transplants from WKY to neonatal SHR will attenuate the development of hypertension (Norman et al., 1986). Immunosuppressive treatment with cyclophosphamide will also attenuate hypertension in SHR (Khraibi et al., 1984).

Increases in sympathetic nervous system (SNS) activity as well as other abnormalities in catecholamine metabolism have been noted in SHR as well as Dahl strain rats (Norman and Dzielak, 1986; Borkowski and Quinn, 1984; Judy et al., 1976; Kuchel et al., 1987; Racz et al., 1987). The SNS has been shown to influence the immune system (Irwin et al., 1988; Braun et al., 1985). This influence tends to be suppressive in nature (Irwin et al., 1988; Braun et al., 1985). The SNS innervates immune organs such as spleen, lymph nodes and thymus. This innervation tends to be concentrated in areas high in developing T-cells but avoids those areas with high B-cell populations (Bulloch, 1985). Interestingly, there was a general decrease in the T-cell population during our study but an increase in the percentage of B-cells. This would tend to support the hypothesis that the SNS is in some way related to this alteration in the lymphocyte populations.

Salt sensitive Dahl rats, as well as SHR, have increased AVP levels (Crofton et al., 1978; Matsuguchi et al., 1981). AVP has been shown to have immunoregulatory properties. AVP receptors are found on T-lymphocytes. These receptors may be involved in the production and secretion of various lymphokines (Johnson and Torres, 1985). AVP immunoreactivity is found in the thymus and lymph nodes (Markwick et al., 1986; Aravich et al., 1987). Also, rats that are genetically AVP deficient show an increase in natural killer cell activity (Yirmiya et al., 1989). DS rats have higher AVP binding in the spleen, an important organ in the immune system (Thibonnier et al., 1986). It thus appears that this increase in AVP could be modulating some of the differences we have observed.

Dahl salt sensitive and S/JR have also been shown to exhibit alterations in steroid production. Aldosterone is decreased in the salt-sensitive animals (Baba et al., 1986) while 18 OH deoxycorticosterone, a mineralocorticoid, is increased relative to the salt resistant variety (Gomez-Sanchez and Gomez-Sanchez, 1988; Baba et al., 1986, Wohlfeil et al., 1988). Plasma corticosterone has been shown to be decreased in DS rats (Rapp and Dahl, 1971; Rapp and Dahl, 1972), but in another study urinary excretion of corticosterone in S/JR rats was increased (Gomez-Sanchez and Gomez-Sanchez, 1988). Urinary excretion normally indicates higher production however a difference in the rate of metabolism can alter the rate of excretion. This observation may also reflect a difference between the original Dahl S rats and the inbred S/JR. Adrenal steroids have immunoregulatory properties. These steroids tend to inhibit cell mediated immunity more than humoral immunity. Abnormalities in steroid production cannot be overlooked as a possible contributor to the alterations in lymphocyte populations we have observed.

It is interesting to speculate about the increase in B-cells noted in the S/JR rats. In adult SHR we have found a decrease in the amount of B-cells (Chapter 4). In rats chronically infused ICV with Ang II, we also see a decrease in the percentage of B-cells (Chapter 3). SHR have higher brain levels of Ang II (Phillips and Kimura, 1988). In the S/JR animals we see no such difference in brain Ang II (unpublished data). Peripherally, DS rats have a decreased plasma renin activity and decreased aldosterone levels (Baba et al., 1986). Two angiotensin converting enzyme (ACE) inhibitors, lisinopril and enalapril, will lower blood pressure in Dahl S rats although the enalapril may be exerting its effect through actions other than those of an ACE inhibitor (Sharma et al., 1983; Fernandez et al., 1988).

It is possible to hypothesize that the central renin-angiotensin system may somehow be responsible for this observed decrease in the percentage of B-cells. One possible site of action may be interactions of angiotensin II containing neurons of sympathetic efferent nerves in the medulla.

Lymphocytes, macrophages and other immunocompetent cells produce and secrete various chemical substances through which they communicate with each other and control proliferation and activity of the various cell types. Evidence is accumulating to suggest that these cytokines can act not only locally during an immune response, but can exert many effects throughout the body. The role of these chemical mediators in controlling and regulating the normal body homeostasis remains largely speculative. Because relatively small amounts of cytokines can have large effects on the proliferation and activity of other immunoactive cell types (thymocytes, macrophages, etc), the proportion of one cell type to another is important. The ratio of one cell type to another plays an important role in maintaining the proper chemical milieu in the system. An alteration in these cell ratios may set the stage for disease processes to start, such as hypertension. Our data clearly show alterations in different lymphocyte populations in prehypertensive and hypertensive inbred Dahl rats. Whether or not these changes are the cause of the hypertensive state in the S/JR remains to be definitively proven, but the fact that these alterations are present clearly points to this as an area that deserves further study.

In summary, we show here decreases in the percentage of T-cell populations and an increase in the percentage of B-cell at 1, 2, and 3 months of age in S/JR animals. These changes were found to occur both

before and after the administration of a high sodium diet and in the prehypertensive as well as the hypertensive phases of development.

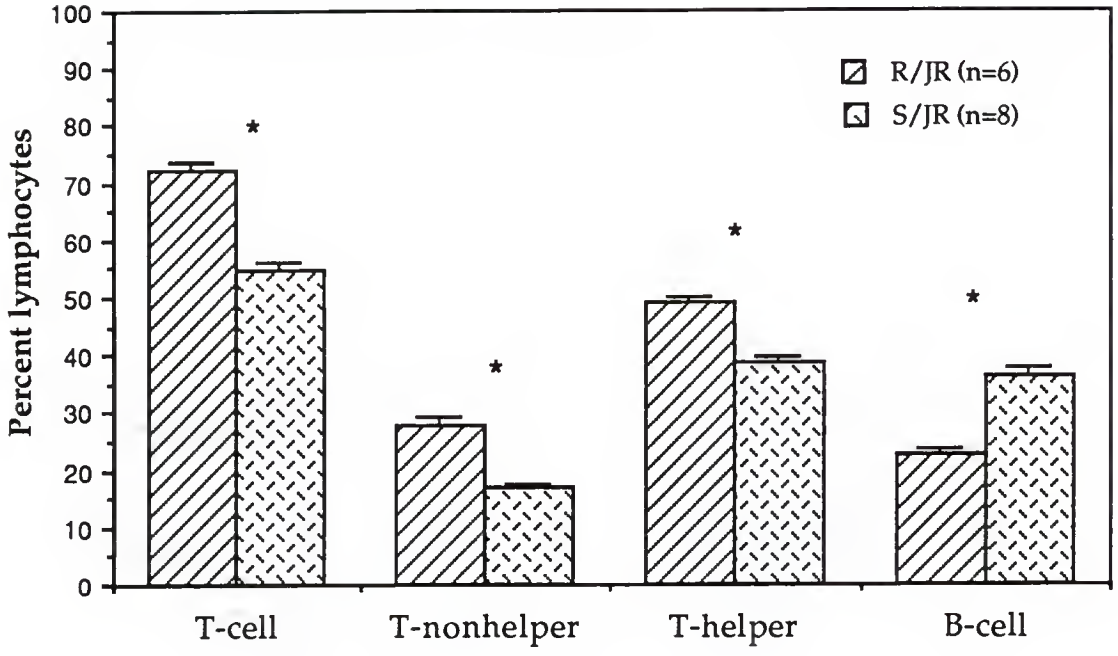

Figure 8-1: Percentage of lymphocyte populations at one month of age in Dahl R/JR and S/JR rats. These values were obtained immediately prior to placing the animals on an 8% NaCl diet. All values are Mean ± SEM.
* = $p<0.05$.

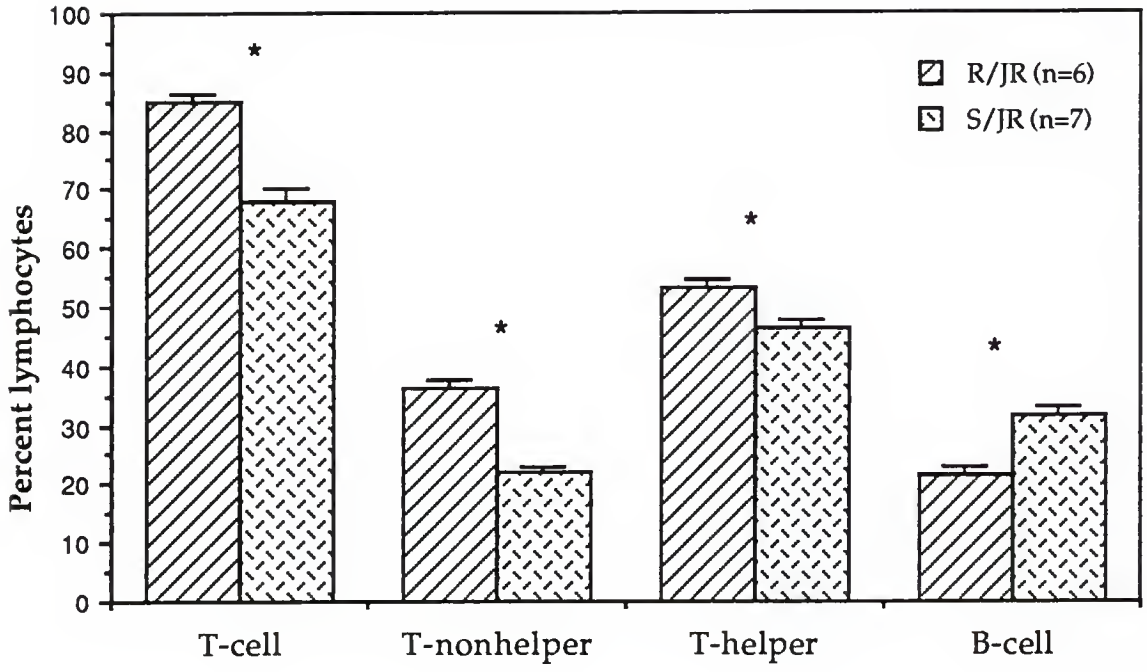

Figure 8-2: Percentage of lymphocyte populations at two months of age. All animals have been on the high sodium diet for one month. All values are Mean ± SEM. * = $p<0.05$.

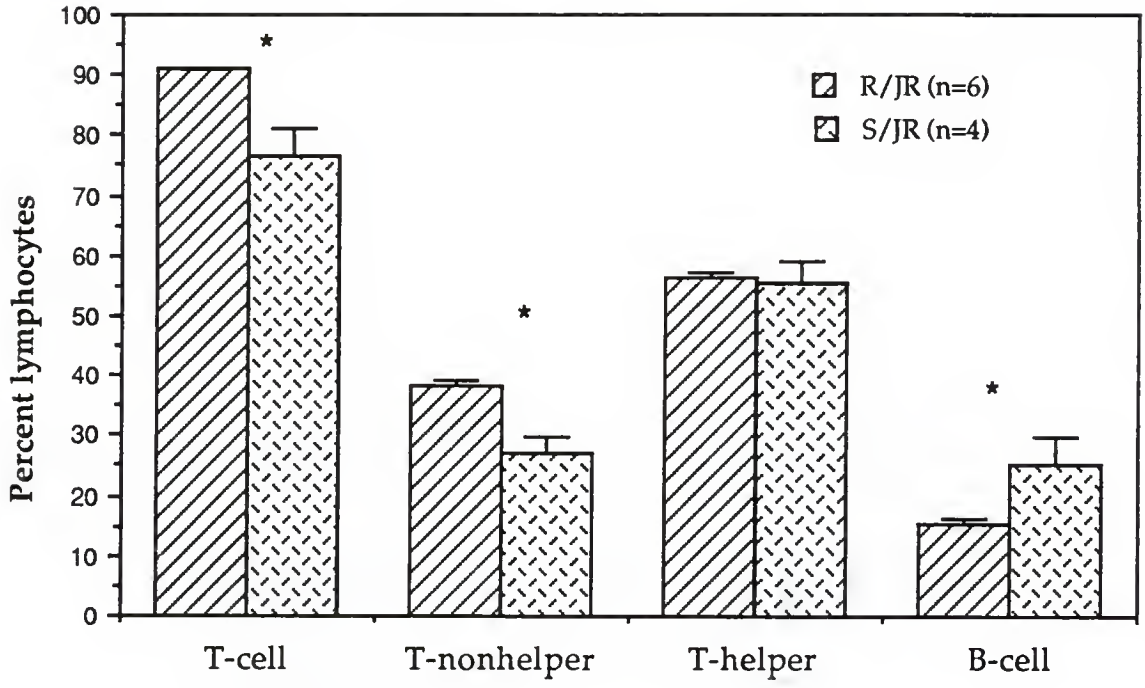

Figure 8-3: Percentage of lymphocyte populations at three months of age. All animals have been on the high sodium diet for two months. All values are Mean ± SEM. * = $p<0.05$.

TABLE 8-1:
SYSTOLIC BLOOD PRESSURES TAKEN AT THREE MONTHS OF AGE
IN R/JR AND S/JR ANIMALS

	N	BP
R/JR	6	135±7.5*
S/JR	7	202±8.6

All values are Mean ± SEM. * = $p<0.05$ for R/JR vs S/JR

CHAPTER 9
TOOTH LOSS AND HYPERTENSION IN THE SPONTANEOUSLY HYPERTENSIVE RAT

Introduction

The Spontaneously Hypertensive Rat (SHR) has been considered a useful model for studying essential hypertension. Hypertension in these rats develops after four weeks of age and becomes permanent and stable at 4-6 months. The hypertension has been correlated with numerous abnormalities compared to normotensive controls. However, the primary cause or causes of this hypertension are unknown. The SHR has an increased sympathetic nervous system (SNS) activity (Norman and Dzielak, 1986; Iriuchijima, 1973; Judy et al., 1976; Baird, 1977; Norman and Dzielak, 1982) as well as other more subtle changes involving CNS peptides (Phillips and Kimura, 1988) which may contribute to the hypertensive state. Alterations in the immune system of the SHR have also been linked to their development of hypertension (Norman and Dzielak, 1986; Svendsen, 1979; Fernandes et al., 1986). Several studies have shown abnormalities in calcium metabolism in the SHR (Izawa et al., 1985; Stern et al., 1984; Roullet et al., 1989; Lucas et al., 1986). Among these abnormalities in the SHR are a decreased intestinal transport of calcium (Roullet et al., 1989; Lucas et al., 1986), decreased serum ionized calcium (Stern et al., 1984) and reduced 1, 25 dihydroxyvitamin D3 (Lucas et al., 1986). All of these possible mechanisms could lead to alterations in

bone density. Few studies on the link between hypertension and bone condition have been reported. Decreases in bone calcium levels and mean cortical bone thickness have been observed(Izawa et al., 1985). Oral manifestations, however, have not been studied in SHR. Large numbers of patients seen by their dentist are hypertensive. Very little attention has been given to the role of hypertension and cardiovascular disease in the development of oral pathology. Recently a significant association has been made in patients linking poor dental health to acute myocardial and cerebral infarctions (Mattila et al., 1989; Syrjanen et al., 1989). In these studies the roles of high sucrose in the diet, socioeconomic level, smoking and other factors could not be differentiated. In a rat model of hypertension these factors can be ruled out. Although numerous studies have looked at a multitude of factors that correlate with the hypertensive state of the SHR, none that we are aware of has been directed at the hard tissues of the oral cavity. Therefore, we examined the hard tissue of the oral cavities of the SHR as a rodent model of hypertension and report here a significant precocious loss of molars and periodontal bone support in the SHR compared to normotensive control rats.

Purpose

The goal of this study was to see if SHR exhibited alterations in the hard tissues of the oral cavity relative to two other normotensive strains.

Rationale

SHR exhibit an increased sympathetic nervous system activity as well as heightened response to stress. Stress has been shown to have a large impact on the health of the oral cavity in humans and other experimental animals. Since stress and the immune system are related we therefore continued the theme of this thesis by analyzing long term effects on teeth.

Methods

Mature, male SHR, WKY and Sprague-Dawley rats were obtained from Charles River Labs, Inc. (Wilmington, MA). Rats were kept on an ad lib standard rat chow diet (#5001, Purina Mills, St. Louis) and tap water. All rats were housed individually and the room was maintained at constant temperature on a 12 hour light-day, 12 hour dark-night cycle. Immediately prior to sacrifice, both body weight and blood pressure were measured. Systolic blood pressure was measured indirectly in an unanesthetized state using a tail plethesmograph. The animals were sacrificed by anesthetic overdose at 6-8 months of age. Mandibles and maxillas from the SHR (n=8), WKY (n=9) and Sprague-Dawley (n=5) rats were dissected out. Radiographs of the jaws did not provide sufficient resolution in anesthetized rats so that tissue had to be dissected and as much tissue as possible removed from the bone. No missing teeth or gross abnormalities were noted in the maxilla so further investigation was limited to the mandible. The mandibles were then hemisected and placed in a concentrated papain solution for several days. All remaining tissue was scrubbed off and the mandibles were placed in a sodium hypochlorite solution for six hours. No molars were lost in this procedure but the incisors were loosened and therefore were removed. The jaws were then dehydrated in 70% ethanol, and were examined under a dissecting microscope. Measurements were obtained with a micrometer of known calibration. Each tooth was systematically checked and the data were recorded on a dental map (Figure 9-1). Crown length was measured on the lingual aspect of the first and third molars from the top of the crown to the cemento-enamel junction (CEJ) as indicated in Figure 9-2. In the case of

the first molars the measurement was made along the long axis of the middle root. For the third molars the measurement was made using the furcation area as a reference point and measured against the long axis of the tooth. Bone loss measurements were again made on the lingual aspect from the CEJ to the top of the crestal bone parallel to the long axis and adjacent to each of the three first molar roots and both of the third molar roots (Figure 9-2). The lingual aspect was used because of the presence of a bony ridge on the buccal surface that made accurate measurements difficult. These values were then summed for each half of the hemisected jaw to form an index of bone loss. This method of measuring alveolar bone loss is the same or quite similar to that used in other rodent models (Kametaka et al., 1989; Crawford et al., 1978; Heijl et al., 1980; Wolff et al., 1985; Messer, 1980; Messer and Douglas, 1980). The data are expressed as mean ± standard error of the mean (S.E.M.). An analysis of variance followed by Newman-Keuls test was used to evaluate the differences between the means of the groups.

Results

Body weights and blood pressure data at the time of sacrifice are given in Table 9-1. All of the SHR were hypertensive at the age tested. The SHR had significantly higher blood pressure than either the WKY or Sprague-Dawley ($p<0.05$). The SHR rats weighed less than their age matched WKY and Sprague-Dawley control animals ($p<0.05$). Examination of the hard tissues of the oral cavity in the SHR revealed that all SHR examined had some evidence of tooth decay, attrition or tooth loss. SHR mandibular molars still present had decay or severe "attrition" not of a carious nature. Of all mandibular second molars, 50% were missing or severely decayed,

with only root tips remaining (Figure 9-3). The control WKY rats and the SD rats presented no tooth decay and had all mandibular molars (Figure 9-4). It was noted that 25% of the SHR had frank pulpal exposures associated with the remaining first and third molars. No evidence of bony repair or recontouring to fill in an empty socket was noted. In every case the missing tooth was a second molar. Crown length determinations revealed that the SHR had significantly shorter third molar crown lengths than either the WKY or SD strains. The SHR right first molar crown length was significantly shorter than that of age matched SD rats (Table 9-2). The right mandible of the SHR showed significantly greater bone loss than either the WKY or SD (Table 9-3).

Discussion

The results show an unequivocal loss of teeth only in the hypertensive rats as well as a greater susceptibility to tooth decay and loss of supporting bone. As with the hypertension in the SHR, the mechanism of decay and bone loss may be related to diet, alterations in calcium metabolism, immune deficiency, and sympathetic innervation of the periodontal vasculature.

We can probably rule out toxins in the diet. SH rats generally weigh less than their WKY age matched controls, including those in this report, due to reduced food intake. Indeed the tendency for early tooth decay might contribute to this food intake reduction. However, lower food intake by SHR compared to WKY would reduce the possibility of a nutritional toxicity in the diet as the cause, because the WKY and Sprague-Dawley animals on the same diet had healthy teeth. There is the possibility of diet deficiency with the reduced food intake but the homogenized lab diet is nutritionally balanced and only the number of

calories would be reduced. Fluoride levels are not a significant cause as all groups received tap water which contains 0.85111 ppm fluoride.

There may be differences in intestinal absorption in the different strains, especially with respect to calcium. It is also possible that there could be differences in the mineral content and make-up of the teeth in these different strains. The SHR molars still present exhibited severe attrition with extensive decay of the anatomical crown. Changes in mineral content in the enamel and dentin are a possible explanation. There is evidence of alterations in calcium metabolism in the SHR (Izawa et al., 1985; Stern et al., 1984; Roullet et al., 1989; Lucas et al., 1986). With a normal diet serum ionized calcium concentrations in SHR have been reported to be 1.58 ± 0.06 mmole/liter compared to WKY 1.91 ± 0.07 mmole/liter (Stern et al., 1984). A few previous studies have reported decreased thickness of cortical bone (Izawa et al., 1985) as well as decreased bone calcium (Lucas et al., 1986) in SHR. It does not seem surprising, therefore, that the teeth and jaws would be affected as well.

Another possible mechanism for the tooth loss in the SHR is immunodeficiency. We have found immune abnormalities associated with age in the SHR. Specifically, SHR of 4 months have a decreased T-cell and B-cell population percentage compared to WKY (Chapter 4). Recently, several studies in humans have looked at lymphocytes, especially the ratios of different lymphocyte subpopulations and their role in the development and maintanence of periodontal pathologies.(Meng and Zheng, 1989; Kinane et al., 1989; Reinhardt et al., 1988a; Reinhardt et al., 1988b). Some of these studies suggest that the ratio of helper to nonhelper T lymphocytes is significant in the periodontal disease process

(Meng and Zheng, 1989; Kinane et al., 1989). These immune system alterations could play a role both in hypertension and periodontal tissues. Immune system abnormalities have been suggested as a causative factor in both periodontal disease and hypertension (Svendsen, 1979; Nisengard, 1977). Immune cells (T and B lymphocytes, macrophages, PMNs) communicate with each other and exert their actions via lymphokines and other chemical mediators. These bioactive immune cell mediators have been associated with bone and tissue destruction in the periodontium (Grant et al., 1979a). Increased periodontal bone loss has been found in rats that have had their lymphocyte populations experimentally altered (Klausen et al., 1989). Permanent T lymphocyte deficiency did not interfere with the development of periodontal disease in pathogen free rats but reduction in total B lymphocytes increased bone loss (Klausen et al., 1989). This fits with our data where B cells are reduced in mature SHR and tooth loss is clear whereas at two months, when tooth loss is not apparent, the B-cell number in SHR is not different from that found in WKY (Chapter 4). Thus alterations in the normal balance of the immune system may lead to both hypertension and tooth loss but T-cells may be more critical in the former and B-cells in the latter.

Another characteristic of the SHR is an overactive sympathetic nervous system (SNS) (Norman and Dzielak, 1986). Increased SNS activity in the gingival circulatory bed could influence this propensity for tooth and bone loss via a mechanism of decreased blood flow to periodontal tissues as well as the teeth. Manhold (1956) hypothesized a connection between the autonomic nervous system and oral pathology. He proposed that increased sympathetic activity could result in a constriction of blood

vessels and thus, a lack of oxygen and nutrients to the teeth and periodontium. However, this has not been demonstrated in the SHR.

Although various characteristics of the hypertensive SHR have been looked at extensively (Norman and Dzielak, 1986; Iriuchijima, 1973; Judy et al., 1976; Baird, 1977; Norman and Dzielak, 1982; Matsunaga et al., 1975; Evan et al., 1981; Borkowski and Quinn, 1984), to our knowledge the data here represent the first report concerning their oral cavity. There appears to be multiple genes that predispose the SHR to express phenotypic hypertension and these genes may contribute to the development of other abnormalities independently of hypertension. However, whether oral changes are secondary or occur independently of hypertension, they may be a clinically important finding in prolonged hypertension. Also of interest is the presence of frank exposures of the pulp chamber in the SHR. If the animals perceive this as a "painful" stimuli it could provide a partial explanation for the observed hyperreactivity of SHR (Hendley et al., 1988; Rettig et al., 1986). As a continuous stressor, tooth pain or discomfort may contribute to the maintainence of hypertension with age. There are many breeders of SHR and we have not tested SHR from more than one source. However, even if there were only one strain of "tooth losing SHR", the finding will be valuable. There are useful subsets of hypertension to be analyzed, for example, "stroke prone SHR"," salt sensitive SHR", "borderline SHR", so that the addition of a "tooth loss SHR" to the repertoire would provide a model for studying the relationship between oral disease and hypertension. The finding may be appropriate for further studies relating diet to hypertension and oral health. In most human studies this has been related to socioeconomic factors. In the SHR these can be excluded. These animals were on a carefully controlled lab diet. If

this condition is the result of an abnormality in the hard tissue matrix of the teeth then modifying diet could accelerate tooth loss. In humans and SHR, alterations in calcium metabolism have been implicated in the pathogenesis of hypertension (McCarron, 1982). In man a weakened matrix of the enamel and dentin would be greatly affected by diet, especially a diet consisting heavily of refined carbohydrates that society often considers normal. If tooth decay or periodontal disease may be a consequence of (or related to) hypertension, then this will be an additional reason for dentists to monitor hypertension in their patients and to educate them.

In summary, we report here a highly significant loss of teeth in mature, adult SHR with established hypertension, which was not seen in WKY or Sprague-Dawley normotensive rats. The data indicate that this loss results from tooth breakdown and an increase in loss of periodontal bone support. The SHR offers a model to study oral health and hypertension. The mechanism for tooth loss remains to be elucidated but calcium loss, impaired immune competence and heightened sympathetic activity which are found in SHR may be contributory factors.

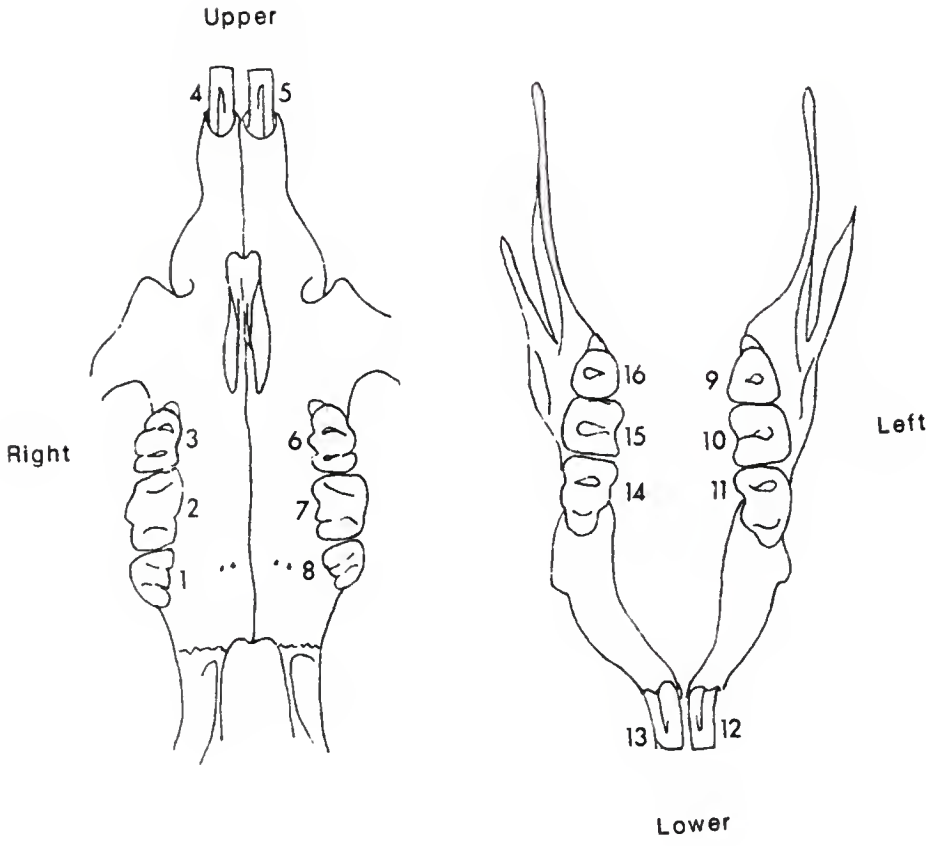

Figure 9-1: Schematic map of the upper and lower jaws of an adult rat. Tooth loss was noted exclusively in the mandibular second molars (#10 and #15).

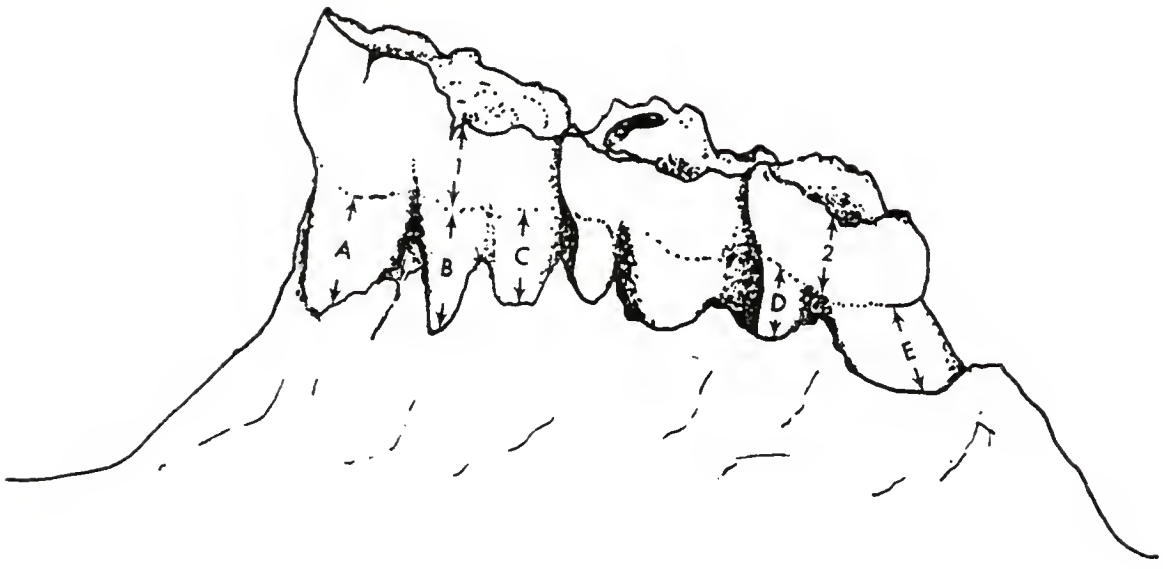

Figure 9-2: Illustration showing method for determining crown height and bone loss measurements. Measurements 1 and 2 were used to determine crown height for first and third molars respectively. Measurements A, B, C, D and E were summed for determination of bone loss index for each hemimandible.

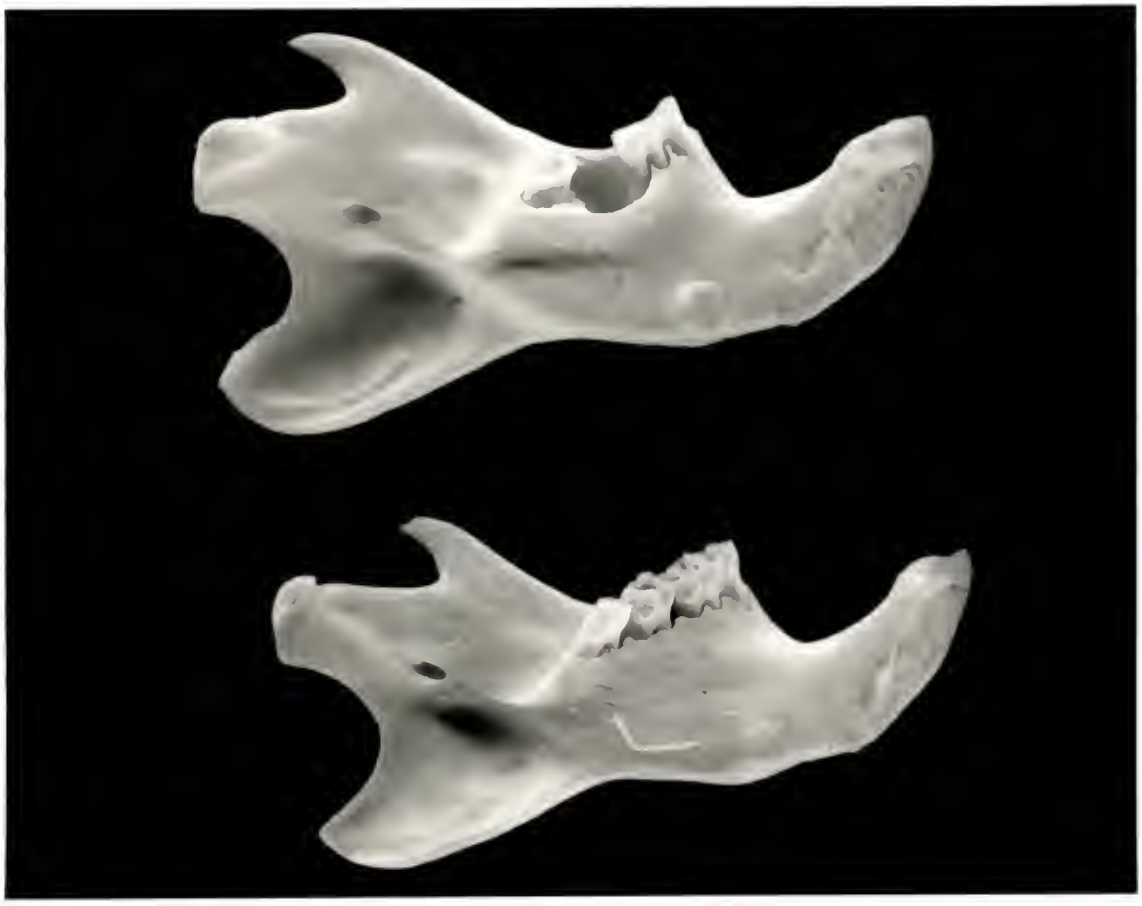

Figure 9-3: A photograph of hemimandibles from SHR (top) and WKY (bottom) illustrating loss of a second molar and the severe bony defect that remains in the SHR.

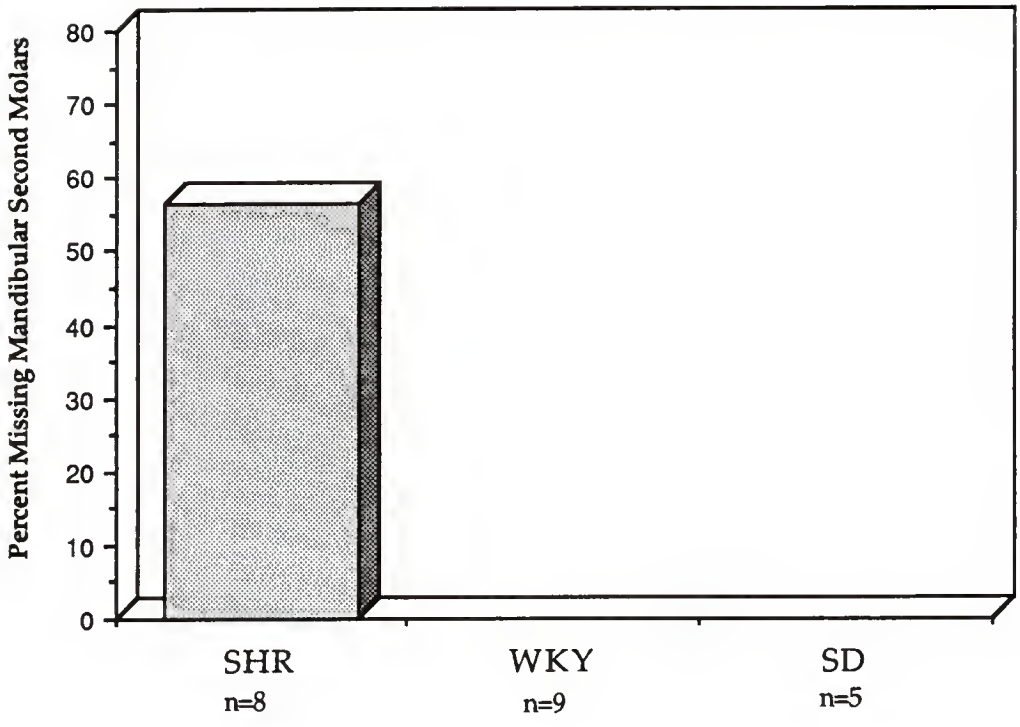

Figure 9-4: Percentage of missing or severely broken down (i.e. only root tips remaining) mandibular second molars in six to eight month old SHR, WKY and Sprague-Dawley rats.

TABLE 9-1:
BODY WEIGHT AND SYSTOLIC BLOOD PRESSURE DATA FOR THE DIFFERENT STRAINS OF RATS EXAMINED

	n	Weight (g)	Systolic blood pressure(mmHg)
SHR	8	380.7±8.4*+	186.3±4.1*+
WKY	9	409.4±8.6	107.4±3.0
Sprague-Dawley	5	542.0±13.9	122.1±2.5

Body weight determinations were made immediately prior to sacrifice. Systolic blood pressure measurements were made within the week preceding sacrifice. All values are Mean ± SEM. *$p<0.05$ for SHR vs WKY. +$p<0.05$ for SHR vs Sprague-Dawley.

TABLE 9-2:
DIFFERENCES IN MANDIBULAR CROWN HEIGHT AMONG VARIOUS RAT STRAINS

	SHR	WKY	SD
Left first molar(mm)	1.75±0.12	1.62±0.05	1.93±0.04
Left third molar(mm)	1.25±0.11*+	1.68±0.05	1.63±0.08
Right first molar(mm)	1.52±0.09+	1.68±0.07	1.97±0.03
Right third molar(mm)	1.32±0.07*+	1.68±0.07	1.73±0.04

Measurements were made from the CEJ to the top of the crown. All values are Mean ± SEM. * $p<0.05$ for SHR vs WKY, + $p<0.05$ for SHR vs Sprague-Dawley.

TABLE 9-3:
BONE LOSS INDEX

	SHR	WKY	SD
Right Mandible(mm)	10.6±0.27*+	9.45±0.22	8.92±0.5
Left Mandible(mm)	10.1±0.45	9.60±0.28	9.65±0.11

Bone loss index indicates the sum of bone lost around each of the three roots of the first molar and each of the two roots of the third molar. Measurements were made from the CEJ to the level of crestal bone adjacent to each root. All values are Mean ± SEM. *$p<0.05$ for SHR vs WKY, +$p<0.05$ for SHR vs Sprague-Dawley.

CHAPTER 10
GENERAL SUMMARY

The major goal of this project was to examine the role of the autonomic nervous system in influencing the immune system. This was achieved using different experimental and genetic models of increased sympathetic nervous system activity. The genetic models of increased SNS activity used in the project, the SHR and Dahl S/JR rat, are also hypertensive. In one of these models, the SHR, the hypertensive state has been suggested to be caused, at least in part, by an alteration of the immune system. Given this fact, part of this project involved giving these animals an immunomodulating chemical in an attempt to reverse the hypertensive condition. Also, in light of the fact that periodontal disease in both humans and experimental animals has an immune system component, the oral cavities of the SHR were examined.

When infused centrally, Ang II and Sub P will increase sympathetic nervous system activity. In chapter 3, we infused Ang II and Sub P into the brains of intact Sprague-Dawley rats for one month and two weeks, respectively. The results indicated an increase in the percentage of total T-lymphocytes for both the Ang II infused animals and the Sub P infused animals. The Ang II infused animals also had a decrease in the percentage of B-cells. It is possible to speculate that this decrease in B-cells may result from the fact that central Ang II infusion also causes an increase in AVP secretion that does not occur with Sub P infusion. This experiment

showed that centrally active peptides can influence the peripheral immune system.

The SHR is a genetic model of increased sympathetic nervous system activity as well as hypertension. Immune abnormalities have been suggested by many investigators as a factor in the development of the hypertensive state. This study looked at different lymphocyte populations in both the prehypertensive and hypertensive phases of development. If changes in lymphocyte populations are noted in the prehypertensive phase of development then this would lend support to the idea that the immune system is involved in this disease state. Our results showed alterations in the percentages of certain lymphocyte populations from as early as two weeks of age. Specifically, the T-nonhelper cell percentage was reduced in the SHR verses the WKY animals throughout the entire four month study. By four months of age all lymphocyte population percentages were reduced in the SHR.

Chapter 4 demonstrated alterations in lymphocyte populations throughout the entire development of the SHR, a model of increased SNS activity. In chapter 5 the SNS was blocked from birth using guanethidine. At both one month of age and four months of age the percentage of T-nonhelper cells were increased in the guanethidine treated SHR verses the untreated SHR. All of the guanethidine treated SHR were non-hypertensive. These studies demonstrated that by blocking the SNS we restored the percentage of T-nonhelper cells and produced a non-hypertensive SHR.

A recent study by one group showed that interleukin-2 given to prehypertensive SHR could abolish the development of hypertension. IL-

2 is involved in the proliferation of activated T-lymphocytes. SHR have been shown to posses a depression of T-lymphocyte function. To further lend credence to this idea, thymic implants from WKY into neonatal SHR will attenuate the development of hypertension. The thymus is responsible for the development of T-lymphocytes. We were unable to replicate the results previously mentioned. The IL-2 treated SHR in our study had blood pressures no different from the untreated SHR. What we did notice, however, was that the IL-2 treated SHR did show an increase in the percentage of T-nonhelper cells. It is interesting to speculate why, given the apparent restoration of the T-nonhelper population that we saw no decrease in BP. The IL-2 was given at 42 days of age. Perhaps the damage had already been done by this point or the IL-2 we used was less active than that used by Tuttle and Boppana (1990).

The spleen is an important organ in the immune system. It is a site of lymphocyte proliferation as well as a site of release of many immunocompetent chemical substances. Several investigators have found Ang II binding sites in the rat spleen as well as isolated rat spleen cells. It is also known that macrophages can secrete Ang II. In Chapter 7 we looked at actual levels of Ang II in rat spleens. The results revealed relatively high levels of Ang II in these spleens. Considering the function of the spleen, it is certainly reasonable to hypothesize a role for Ang II in the immune reactions occurring there.

A further aim of this project was to examine the lymphocyte population dynamics in different genetic models of increased sympathetic nervous system activity. The Dahl S/JR rat was chosen for this purpose. This strain will become spontaneously hypertensive, but the rate of development of hypertension is greatly increased when it is placed on a

high sodium diet. Our results showed alterations in the S/JR animals from one month of age. Specifically, all T-cell populations were decreased and there was an increase in the percentage of B-cells found. Interestingly, this model, like the SHR, exhibited a decrease in the nonhelper percentage in the prehypertensive state.

Both of the genetically hypertensive models used in this study, the SHR and the Dahl S/JR rat share similar characteristics. Besides the increase in sympathetic nervous system activity (Judy et al., 1976; Kuchel et al., 1987) another characteristic shared by these inbred lines is an increase in AVP secretion (Crofton et al., 1978; Matsuguchi et al., 1981). The renin-angiotensin system also appears to play a role in both strains. SHR have an increased level of brain angiotensin II (Phillips and Kimura, 1988). Various ACE inhibitors will decrease blood pressure in these animals. Two ACE inhibitors, enalapril and lisinopril also will reduce blood pressure in S/JR rats, indicating a role of the renin-angiotensin system in this model as well, although the exact mode of action of these drugs in the S/JR strain is not completely understood (Sharma et al., 1983; Fernandez et al., 1988). Given these similarities it does not seem surprising that both strains of rat should also share some immunological characteristics as well.

The final aim of the project was to use the SHR as a model of both increased SNS activity and hypertension and examine this model for any changes occurring in the oral cavity. The SHR, as mentioned previously, appears to have several immune system abnormalities. Among these is a decrease in the activity of T-suppressor cells. Decreases in the activity of this cell type are often linked to autoimmune disorders. Along these same

lines, human periodontal disease is believed to have an autoimmune component. The SHR rats that we examined showed a greater tendency for tooth and bone loss than either the WKY or Sprague-Dawley strains. This study points to the SHR as a possible model of periodontal disease that may more accurately reflect the human condition.

All of our experiments have a central underlying theme, namely, that the autonomic nervous system can influence immune function and, thus, our susceptibility to disease, two examples being hypertension and periodontal disease. The autonomic nervous system is truly a mirror of our emotional health and well being. The importance of this should be obvious. Our emotional health is directly related to our daily lives. Life is relationships. These relationships include our relationship with ourselves, our relationships with others and also our relationships with our environment and our physical surroundings. In today's society, often times these relationships are not very positive in nature. Once it is established that this connection exists perhaps it will be possible to restructure our lives to promote better emotional health and, from this, better physical health. This all returns to the basic ideal goal: that from studies along these lines we can begin to address the cause of disease instead of just treating the symptoms.

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APPENDIX

Number of Fluorescently Labeled Cells per 10,000 Cells Counted

SHR and WKY Age Experiment

Two weeks

	T-cells	T-nonhelper	T-helper	B-cell
WKY				
#1	6577	1515	4670	3341
#2	5141	1432	3752	2872
#3	4155	1259	2820	2662
Mean±SD	5291±1218	1402±131	3747±925	2958±348
SHR				
#3	5000	975	3482	2504
#4	4252	785	3130	2038
#6	4463	1010	3207	2349
#7	4135	985	3126	2286
Mean±SD	4463±383	939±104	3236±168	2294±194

One month

	T-cells	T-nonhelper	T-helper	B-cell
WKY				
#50	5379	1363	3894	2393
#51	5553	1304	4267	2216
#52	5515	1487	3966	2074
#53	5710	1195	3934	1890
#54	5957	1681	4460	1526
#55	6793	1720	4549	1852
#56	6566	1975	4620	1165
Mean±SD	5839±425	1532±274	4241±310	1874±419
SHR				
#58	5972	1246	4875	1845
#59	4946	584	3355	2224
#60	5690	1131	4347	2189
#61	5637	927	4195	1726
#62	5889	985	4357	2264
#63	5849	796	4514	1926
#64	5919	1232	4689	2024
#65	5877	1233	4844	1841
Mean±SD	5722±334	1017±240	4397±487	2004±202

Two months

	T-cells	T-nonhelper	T-helper	B-cell
WKY				
#52	6936	2727	4866	1663
#53	6511	2901	4677	2049
#54	6479	2621	4537	1890
#55	6305	2301	4550	2043
#56	6468	2851	4442	1802
#57	6147	2327	4210	2142
Mean±SD	6474±265	2621±257	4547±221	1931±180
SHR				
#60	6843	2255	5018	1504
#61	6304	2241	4734	1787
#62	6443	2043	4829	1896
#63	6198	1992	4562	2003
#64	6452	2261	4895	1597
#65	6603	2209	4979	1558
Mean±SD	6474±228	2167±118	4836±169	1724±202

Three months

	T-cells	T-nonhelper	T-helper	B-cell
WKY				
#50	6798	2640	4431	1647
#51	6809	2545	4556	1456
#52	6351	2540	4354	1320
#53	6311	2472	4215	1441
#54	6371	2265	4431	1248
#55	6644	2328	4558	1197
#56	6583	2375	4464	1113
#57	6281	2215	4353	1178
Mean±SD	6519±218	2422±150	4420±114	1325±179
SHR				
#58	6225	2046	4246	1206
#59	5988	1901	4229	1338
#60	5890	1905	3929	1138
#61	5775	1837	3977	1084
#62	5800	1888	3898	1122
#63	5818	1924	4600	-----
#64	6347	1801	4506	779
#65	5872	1679	4169	690
Mean±SD	5964±212	1873±106	4194±260	1051±233

Four months

	T-cells	T-nonhelper	T-helper	B-cell
WKY				
#50	7280	2865	4528	1381
#51	7008	2738	4609	1383
#52	6939	2786	4726	1306
#53	7027	2617	4877	1335
#54	7031	2935	4652	1652
#55	6830	2719	4584	1547
#56	6515	2488	4468	1452
#57	6565	2202	4214	1533
Mean±SD	6899±255	2669±234	4582±194	1449±120
SHR				
#58	5778	2165	3743	1042
#59	5745	2115	3759	1082
#60	5734	1887	3984	1183
#61	5721	1816	4018	1071
#62	5781	1936	3779	1139
#63	5719	1928	3910	1237
#64	5470	1961	3449	1234
#65	5736	1983	3904	1232
Mean±SD	5710±100	1974±115	3818±181	1153±80

Dahl Rat Experiment

One month

	T-cells	T-nonhelper	T-helper	B-cell
R/JR				
#95	5678	2435	3828	1860
#96	5302	2022	3434	1794
#98	6168	2480	4194	1699
#99	5713	2185	4185	1992
#101	5997	2215	4354	1995
#102	6371	2473	4352	1598
Mean±SD	5872±385	2302±189	4058±361	1826±159
S/JR				
#87	4260	1382	3006	2913
#88	4139	1294	2941	2901
#89	4619	1549	3428	2460
#90	4987	1543	3665	2479
#91	4113	1256	3112	3025
#92	4620	1179	3316	3312
#93	4602	1483	3253	2440
#94	4407	1368	3167	2813
Mean±SD	4468±296	1382±136	3236±235	2793±313

Two months

	T-cells	T-nonhelper	T-helper	B-cell
R/JR				
#95	7131	3258	4551	1875
#97	7534	3459	4392	1905
#98	7390	2882	5032	1254
#99	7078	3083	4823	1776
#101	6822	2918	4557	2179
#102	6997	3170	4403	1797
Mean±SD	7159±261	3128±217	4626±252	1798±303
S/JR				
#88	4877	1824	3266	2682
#89	5572	1962	3857	2534
#90	5021	1548	3847	2893
#91	6415	2136	4597	2464
#92	6116	2075	4307	2669
#93	5835	1801	3991	2582
#94	6080	1986	4238	2349
Mean±SD	5702±578	1905±199	4015±426	2596±175

Three months

	T-cells	T-nonhelper	T-helper	B-cell
R/JR				
#95	7631	3093	5034	1213
#97	8112	3670	5308	1363
#98	8098	3721	5162	1224
#99	7997	3516	4634	1657
#101	7831	3580	5438	1485
#102	8131	3202	5071	1390
Mean±SD	7967±199	3464±257	5108±277	1389±167
S/JR				
#88	5853	1855	4286	1099
#90	6227	2549	4615	1870
#91	5085	1676	3488	2851
#94	6237	2438	5008	1637
Mean±SD	5851±541	2130±429	4349±646	1864±733

Angiotensin II and Substance P Infusion Experiment

	T-cells	T-nonhelper	T-helper	B-cells
Ang II infused animals				
#70	6952	2547	4702	1398
#74	6573	2995	4026	1567
#77	6402	3270	3674	662
#78	7019	2693	4781	1011
#82	6497	3077	4119	1029
Mean±SD	6689±279	2916±293	4260±470	1133±336
Artificial CSF infused animals				
#66	6536	2505	4403	1938
#71	6741	2991	4431	1528
#75	6619	3043	4097	1630
#79	6149	2098	3997	1402
#83	5268	1920	4063	1729
Mean±SD	6263±535	2511±508	4198±203	1645±203

	T-cells	T-nonhelper	T-helper	B-cells
Sub P infused animals				
#5	6763	2254	5375	1725
#6	6348	2337	3757	2412
#7	7399	3168	5977	2093
#8	7672	2143	4853	2285
#9	6255	2184	4915	2082
#10	5996	2093	4055	2103
Mean±SD	6739±670	2363±403	4822±821	2117±233

Artificial CSF infused animals				
#1	6246	1896	5268	2166
#2	6339	2170	4581	2556
#3	5459	1895	4330	2650
#4	6197	2045	4848	2242
Mean±SD	6060±405	2002±133	4757±401	2404±236

Guanethidine Experiment

One month

	T-cells	T-nonhelper	T-helper	B-cell
WKY				
#1	5714	2044	4220	2378
#2	5588	1821	4347	2338
#3	5002	1461	3942	2289
#4	5479	1652	4435	1752
#5	5273	1480	3940	2419
#6	6194	1948	4918	1730
Mean±SD	5542±406	1734±243	4300±365	2151±320
SHR				
#7	5768	1371	4633	2544
#8	5644	1369	4425	1909
#9	5726	1330	4548	1208
#10	5667	1523	4352	1694
#11	5277	1411	3830	1943
#12	5450	1356	4052	1825
Mean±SD	5589±188	1393±69	4307±308	1854±431
Guan SHR				
#1	5458	1995	4329	1824
#2	5596	1663	4427	1699
#3	5144	1441	4500	1339
#4	5224	1458	4400	1264
#5	5946	1682	4754	1322
#6	5754	1511	4524	1567
Mean±SD	5520±308	1625±208	4489±148	1503±229

Three months

	T-cells	T-nonhelper	T-helper	B-cell
WKY				
#1	7015	-----	6286	1945
#2	7061	2750	6315	1638
#3	6081	2036	4515	2227
#4	5857	1891	4191	2314
#5	6782	2469	4460	2307
#6	6860	2234	4526	3114
Mean±SD	6609±511	2276±343	5049±977	2258±494
SHR				
#7	6114	1812	4288	1843
#8	6612	1787	4872	1603
#9	5907	1699	4005	1830
#10	6131	1755	4495	1544
#11	6514	-----	4993	2147
Mean±SD	6256±296	1763±49	4531±408	1793±238
Guan SHR				
#1	6024	1529	4899	1093
#2	6465	1650	5102	1086
#3	5803	1356	4735	1321
#4	7451	2624	5077	1161
#5	6018	1872	4711	1483
#6	5792	1775	4871	1420
Mean±SD	6259±633	1801±442	4899±165	1261±171

Four months

	T-cells	T-nonhelper	T-helper	B-cell
WKY				
#1	6263	2222	6901	2184
#2	6842	2114	6818	2133
#3	7076	2119	5029	1893
#4	7538	2053	6327	1798
#5	7823	2221	5801	1027
#6	7828	2052	6323	1156
Mean±SD	7228±619	2130±76	6199±698	1699±493
SHR				
#7	7211	-----	7074	1694
#8	6757	1589	7276	1492
#9	7620	1091	5472	1886
#10	8062	939	6463	1543
#11	7849	1693	6066	1625
Mean±SD	7499±521	1328±369	6470±737	1648±154
Guan SHR				
#1	6619	2211	5602	1821
#2	7127	2223	5791	1524
#3	7325	2451	5862	1300
#4	7135	2360	5424	1589
#5	7252	2464	5759	1857
#6	7192	2258	5376	1921
Mean±SD	7108±251	2328±113	5636±202	1669±239

Interleukin-2 Experiment

Three months

	T-cells	T-nonhelper	T-helper	B-cell
WKY				
#1	7015	-----	6286	1945
#2	7061	2750	6315	1638
#3	6081	2036	4515	2227
#4	5857	1891	4191	2314
#5	6782	2469	4460	2307
#6	6860	2234	4526	3114
Mean±SD	6609±511	2276±343	5049±977	2258±494
IL-2 WKY				
#9	7218	2115	5051	1730
#10	6690	2146	4627	1821
#11	7561	-----	6257	2550
#13	6518	2070	4857	2307
#14	6694	2160	4762	2082
#15	7173	2132	5583	1570
#16	7011	2175	5385	1686
Mean±SD	6981±368	2133±37	5217±571	1964±361
SHR				
#7	6114	1812	4288	1843
#8	6612	1787	4872	1603
#9	5907	1699	4005	1830
#10	6131	1755	4495	1544
#11	6514	------	4993	2147
Mean±SD	6256±296	1763±49	4531±408	1793±238

IL-2 SHR				
#1	7002	2128	5204	1513
#2	6713	2011	4917	1537
#3	7419	------	5139	1557
#4	7053	------	4963	1747
#5	6851	1992	4921	1671
#6	6292	1823	4741	1645
#7	6182	1695	4981	1270
#8	6868	1856	5581	1285
Mean±SD	6798±404	1918±155	5056±255	1528±173

Four months

	T-cells	T-nonhelper	T-helper	B-cell
WKY				
#1	6263	2222	6901	2184
#2	6842	2114	6818	2133
#3	7076	2119	5029	1893
#4	7538	2053	6327	1798
#5	7823	2221	5801	1027
#6	7828	2052	6323	1156
Mean±SD	7228±619	2130±76	6199±698	1699±493
IL-2 WKY				
#9	6914	2010	4758	1785
#10	6665	1835	4697	1933
#11	6841	2020	-----	1973
#12	7171	2248	5323	1581
#13	6795	1828	5324	1658
#14	7610	2117	5402	1402
#15	7748	1911	5519	1730
#16	8107	2071	5361	1809
Mean±SD	7231±527	2005±144	5198±328	1734±187
SHR				
#7	7211	----	7074	1694
#8	6757	1589	7276	1492
#9	7620	1091	5472	1886
#10	8062	939	6463	1543
#11	7849	1693	6066	1625
Mean±SD	7499±521	1328±369	6470±737	1648±154

IL-2 SHR				
#1	7067	1822	5234	1398
#2	6953	1647	5467	1338
#3	7098	2000	5379	1445
#4	7087	2039	-----	1537
#5	7484	1879	5609	1475
#6	7571	1654	5749	-----
#7	6999	1391	5282	1489
#8	7466	1613	5590	1620
Mean±SD	7216±248	1756±219	5473±187	1472±92

BIOGRAPHICAL SKETCH

Lewis D. Fannon was born on June 14, 1961, in Newport Beach, California, where he lived for the first 18 years of his life. He attended college at the University of the Pacific, in Stockton, California. He then moved to Omaha, Nebraska, to attend the Creighton University School of Dentistry, from which he graduated in 1986 with the degree Doctor of Dental Surgery. He then moved on to the University of Florida and entered the graduate program in physiology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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